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Angiogenesis in Inflammation: Mechanisms and Clinical Correlates

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Preface

This book brings together a variety of subjects all directly related to the processes of angiogenesis in inflammation. Whilst angiogenesis in cancer is a well defined field, the processes involved in inflammatory angiogenesis have both differences and similarities. For this reason we have set out to provide a resource detailing the relationship between components of the inflammatory process and the angiogenesis that occurs in chronic inflammation and disease.

The collection is arranged according to various cells and systems that are involved in inflammation and immunity and the role they play in the angiogenic process. Some of these would not normally be ascribed to angiogenic processes, but illustrate the that inflammatory angiogenesis is not as a result of one cell or stimulus, but a culmination of inflammatory cellular and molecular responses. This is tied together with two chapters on the final *in vivo* consequences of angiogenesis, namely chronic inflammatory responses. There are many *in vitro* and other simple *in vivo* systems described in the literature for studying pure angiogenesis, but not so many in the inflammatory milieu. In fact the presence of inflammation in such systems is considered an unwanted and confounding artefact. So we felt it important to included the modelling of inflammatory angiogenesis for research and drug discovery, and more importantly rheumatoid arthritis as a clinical manifestation of chronic inflammatory disease, in which angiogenesis plays a profound role not just in the development of pannus, but its erosive and debilitating consequences.

This collection is only the tip of the research in this area, and we hope will encourage wider reading and interest, and stimulate research into this expanding and highly relevant topic of research and investigation into new therapeutics. It will be of great interest to follow this subject into the next decade, and see the results applied to the clinic.

The editors wish to thank all of the contributors to this volume, such works of scholarship require great dedication and application in the busy modern world of research, as well as the series editor M. Parnham and Birkhäuser for their help, patience, and allowing the opportunity for publishing this book.

April 2008

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Neurogenic angiogenesis and inflammation

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New blood vessel formation

Blood vessels can be formed through two different mechanisms: vasculogenesis or angiogenesis [1]. The first mechanism is dependent on the development of blood vessels from immature mesenchymal cells, and has been traditionally thought to be restricted to the early stages of embryo development. Angiogenesis is the sprouting of new blood vessels from pre-existing ones. This may occur physiologically during the female reproductive cycle or pathologically during tumour growth, diabetic retinopathy and chronic inflammation. It may be beneficial, for example during wound repair, or detrimental, for example in tumours or retinopathies. One of the regulators that may control angiogenesis is the nervous system. Neuropeptides are known to have angiogenic effects *in vitro* and *in vivo*. Neuropeptides are released in acute inflammatory responses but their role during chronic inflammation is much less certain. There appears to be a depletion of nerves in tissues as they become chronically inflamed. This may be related to the inability of nerves to grow at the same rate as proliferating tissue or due to a direct toxic effect of one or more components of the inflammatory milieu. Under such conditions we postulate that other peptides may take over the angiogenic roles of neuronally derived peptides, sometimes utilising the same receptors on endothelial cells. As well as promoters of angiogenesis, there is also a group of neuropeptides that are becoming increasingly recognised as being anti-angiogenic. Peptides released from the peripheral terminals of nerves may therefore either facilitate or suppress tissue growth.

Neurogenic inflammation

Stimulation of unmyelinated sensory nerve fibres produces a local response termed 'neurogenic inflammation', which is characterised in the skin by the wheal and flare reaction [2]. An 'axon reflex' is also proposed whereby activation of sensory nerve

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fibres following tissue insult results not only in impulse transmission to the central nervous system but also reverse transmission through the extensive arborisations of nerve fibres, which follow and terminate near blood vessels. This neurogenic inflammation is mediated by biologically active neuropeptides [3]. These peptides are synthesised in the small and medium-sized dorsal root ganglion cell bodies and are transported *via* unmyelinated sensory fibres to the peripheral tissues and centrally to synaptic terminals within the superficial laminae I and II of the dorsal horn of the spinal cord. Neuropeptides such as substance P (SP), calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP) and somatostatin (SOM) have all been reported as being present in sensory nerve fibres.

SP and the tachykinins

SP is one of the tachykinin family of neuropeptides that share a common carboxyl terminal sequence –FXGLM through which they bind to the neurokinin group of cell surface, G protein-coupled receptors, NK_1, NK_2 and NK_3 [4, 5]. The classic mammalian tachykinins are SP, neurokinin A (NKA) and neurokinin B (NKB) and are encoded by the *TAC1* and *TAC3* genes that are predominantly expressed in nervous tissues. Both SP and NKA are expressed by fine unmyelinated and thinly myelinated sensory nerves. SP, NKA and NKB have relative selectivities and high affinities for NK_1, NK_2 and NK_3 receptors, respectively [6]. SP can induce angiogenesis through direct activation of NK1 receptors [7, 8].

SP *in vitro* effects

In vitro SP can increase proliferation of endothelial cells from a variety of sources, including bovine aorta, human umbilical and coronary venules. This enhanced proliferation is mimicked by selective NK_1 agonists and inhibited by NK_1 antagonists. SP and NK_1 receptor agonists also enhance endothelial cell migration [9] and the formation of tubules by endothelial cells in collagen gels [10, 11]. The proliferation and migration effects appear to be mediated by nitric oxide (NO) since they are blocked by inhibitors of NO synthase [6]. NO donors and SP also up-regulate the production of the angiogenic factor fibroblast growth factor 2 (FGF-2) in coronary venular endothelial cells and, correspondingly, immunoblockade of FGF-2 inhibits SP-induced endothelial cell proliferation [12].

In addition to its actions on endothelial cells, SP has been found to have multiple effects on other cell types *in vitro*, which may well contribute to its angiogenic activity *in vivo*. Angiogenic factors that have been identified following stimulation with SP include tumour necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-8, IL-10 and histamine. These factors may be produced by monocytes and macrophages

[13, 14], polymorphonuclear leucocytes [15], neuroglia and astrocytes [16, 17] and mast cells [18].

The NK_2 and NK_3 receptor agonists NKA and NKB have not been found to increase endothelial cell proliferation, migration *in vitro* [8, 9] or angiogenesis *in vivo* [19].

SP *in vivo* effects

SP-impregnated pellets stimulate vascular growth in the rabbit cornea [8]. High doses of SP induced both inflammation and angiogenesis, whereas lower doses induced angiogenesis in the absence of an inflammatory cell infiltrate. SP can also enhance angiogenesis in the subcutaneous sponge granuloma model in the rat [19]. In this model, polyether sponges are inserted beneath the dorsal skin of a rat and pharmacological agents are introduced by means of an indwelling cannula. Angiogenesis is measured by increased fibrovascular growth and as an increase in blood flow, indicated by increased rates of ^{133}Xenon clearance from the sponge. Such experiments show that SP can stimulate angiogenesis *in vivo* but do not give the complete picture. For instance, NK_1 receptor antagonists do not inhibit basal angiogenesis in the sponge granuloma model, in contrast to the inhibition observed following administration of IL-1 receptor antagonist and combined immunoblockade of FGF-2, IL-8 and TNF-α [20]. Other factors that are generated during inflammation may replicate the angiogenic activity of SP. NK_1 receptor antagonists alone do not inhibit synovial angiogenesis induced by the injection of intra-articular carrageenan, but were able to substantially inhibit vascular proliferation when given concurrently with a bradykinin B_2 receptor antagonist [7].

Other angiogenic factors that are generated *in vivo* during inflammation may enhance the effects of SP on vascular growth. SP-induced vascular proliferation is synergised by CGRP, which is co-released with SP following sensory nerve stimulation [21].

Calcitonin gene-related peptide

The calcitonin family of peptides comprises six distinct members: calcitonin, adrenomedullin (AM), amylin, two calcitonin gene-related peptides CGRP1 and CGRP2 (which differ by 3 amino acid residues and have no known important functional differences) and the recently discovered intermedin (ADM_2). CGRP1 is a 37-amino acid peptide with a 7-amino acid residue ring linked by a disulphide bridge between positions 2 and 7 and has an amidated N terminus [22]. Functional CGRP receptors are a heterodimeric 1:1 protein complex composed of the heptahelical calcitonin receptor-like receptor (CRLR) and an accessory protein termed receptor activ-

ity-modifying protein (RAMP1) [23]. RAMPs comprise a family of three single transmembrane proteins [24]. The RAMPs are chaperones for trafficking of CRLR from the endoplasmic reticulum and Golgi apparatus to the cell surface and they determine the glycosylation state and pharmacological properties of CRLR. When co-expressed with RAMP1, CRLR shows a preference for CGRP over AM, but when co expressed with RAMP2 or RAMP3, CRLR interacts with higher affinity for AM [25].

Receptor component protein (RCP) is probably a third component of the CGRP receptor and is thought to couple CRLR to cell signalling pathways [26].

CGRP *in vitro* effects

CGRP has been shown *in vitro* to directly stimulate human umbilical vein endothelial cells to proliferate [27], indicating a possible role in angiogenesis. Subsequent investigations have found CGRP to be pro-angiogenic in human placental development, stimulating endothelial cell proliferation, migration and capillary-like tube formation [28].

Effects on other cell types relevant to angiogenesis have also been reported. Human keratinocyte cell lines are stimulated to produce vascular endothelial growth factor (VEGF) when exposed to CGRP [29]. This may be of particular importance in diseases such as psoriasis where the density of CGRP-containing nerve fibres is also increased [30]. CGRP has been found to have stimulatory effects on monocytes and macrophages. CGRP up-regulates the production of IL-10 [31]. This offers a possible explanation for the inhibition of IL-2 by CGRP since IL-10 suppresses the activity of T_H1 lymphocytes, which produce IL-2. CGRP potentiates the LPS-induced release of another angiogenic cytokine, IL-6, from murine macrophages, an effect which is thought to be mediated by enhanced production of NO and prostacyclin [32].

CGRP *in vivo* effects

The predominant reported effect of CGRP is its action as a potent arterial and venous vasodilator. The vasorelaxation to CGRP can be blocked by the administration of the peptide fragment $CGRP_{8–37}$, a CGRP receptor antagonist, indicating a specific receptor-mediated mechanism. The microvasculature is very sensitive to CGRP. It is the most potent microvascular vasodilator known, 10-fold greater than the prostaglandins and 100–1000-fold greater than other vasodilators such as acetylcholine, 5-hydroxytryptamine and SP [33]. Plasma protein extravasation in response to CGRP has also been demonstrated, whereas a β-adrenoreceptor agonist failed to induce protein leakage despite comparable vasodilator responses, possi-

bly indicating a direct effect of CGRP on endothelial cells [34]. Administration of CGRP improves the survival of experimental skin flaps [35]. This effect was initially thought to be a consequence of increased perfusion due to the known vasodilatory properties of CGRP. However, in a subsequent study, doses of CGRP that did not induce increased blood flow, as measured by laser Doppler flowmetry, still promoted survival of skin flaps [36]. This may be attributable to decreased neutrophil accumulation or promotion of angiogenesis.

Preliminary experiments in our laboratory indicate that administration of intra-articular CGRP increases the endothelial cell proliferation index in the rat synovium in dose-dependant manner [37]. These results require further investigation to determine whether this represents a direct, receptor-mediated effect.

Sympathetic nerves and neuropeptide Y

Having considered the sensory nervous system and its possible contribution to angiogenesis, we should also mention the postganglionic sympathetic nerves, which are in close proximity to the blood vessels of the peripheral circulation. Such nerves contain neuropeptide Y (NPY). NPY is a 36-amino acid neuropeptide present in the brain, adrenal medulla and sympathetic nerves and extraneuronally in endothelial cells. NPY was found in early experiments not to have a proliferative effect on cultured endothelial cells [27]. However, in more recent experiments it has been shown to stimulate endothelial cell adhesion, proliferation, migration and capillary formation [38]. There are five functional human NPY receptors Y1–Y5. Human endothelial cells express Y1 and Y2 receptors and also dipeptidyl peptidase IV (DPPIV). It is thought that activation of Y2 receptors [39], possibly in conjunction with Y5 receptors are responsible for NPY's angiogenic capability. Conversion of NPY to $NPY_{3–36}$ by DPPIV appears to be a prerequisite for angiogenic activity since DPPIV-neutralising antibodies block angiogenic activity [40].

Effects of neuropeptides during chronic inflammation

SP and CGRP contained within the sensory nerve fibres are well placed to initiate angiogenesis in acute tissue injury [41] but a role in chronic inflammation is less certain. Sensory and sympathetic nerves are depleted during chronic inflammation [42, 43]. Nerve growth also progresses more slowly than does that of blood vessels, and so the neovasculature of tumours, skin grafts and arthritic joints are often relatively poorly innervated, despite expressing NK_1 receptors. SP is rapidly metabolised in biological fluids with a half-life in serum of approximately 7 min [44]. Thus, NK_1 receptors on new blood vessels are unlikely to be exposed to endogenous SP *in vivo*.

Hemokinins

Recently a novel class of tachykinins has been found in rodents and man. The *TAC4* gene in mice encodes a single *TAC4* mRNA encoding a 128-amino acid precursor protein that is believed to undergo post-translational processing to yield the decapeptide (or N-terminal extended unadecapeptide) hemokinin-1 (HK1) [45]. In rats, the *TAC4* gene is predicted to encode a 170-amino acid residue precursor that is again processed to an identical HK-1 [46]. The human *TAC4* was found to encode four splice variants (α, β, γ and δ-*TAC4*), predicting four different peptides named endokinin (EK) A, EKB, EKC and EKD [47]. EKA is encoded only by α*TAC4*, whereas EKB is encoded by all four transcripts. EKA and EKB are N-terminal extended forms of human HK-1 and HK-$1_{4–11}$ [4, 47]. These novel tachykinins are predominantly expressed in peripheral tissue rather than by neuronal cells. In particular they have been found to be expressed by macrophages, lymphocytes and endothelial cells, as well as abundant expression in the placenta [4, 45, 48]. Expression of endokinins by endothelial cells may explain previous reports that 'SP' may be expressed by those cells. Other authors have been unable to detect TAC1 expression in endothelial cells and have not found SP-like immunoreactivity in the endothelial cells of joint tissue of either rat or human. Both HK-1 and EKA/B behave as full agonists at NK_1 receptors in mouse, rat and man, in that they induce vasodilatation and plasma extravasation [49]. It is likely that these novel tachykinins, like SP, induce angiogenesis. Biological roles have yet to be definitively allocated to EKC and EKD. The discovery of endokinins and hemokinins raises important questions about the interpretation of previous data obtained using SP, capsaicin and NK_1 antagonists. It had been assumed that tachykinins may be mostly involved in the initiation of angiogenesis, and this may be true for the neuropeptide SP, since new vessels are not immediately innervated [50]. However, if inflammatory or endothelial cells themselves produce tachykinins with activity at NK_1 receptors on the new endothelial cells, then these novel tachykinins may, in addition, maintain angiogenesis in chronic inflammation, and may promote the survival of newly formed vessels [51]. The actions of tachykinins on the vasculature are often potentiated by inflammation and pro-inflammatory cytokines such as IL-1 [52]. The autocrine production of endokinins by endothelial cells raises the possibility that NK_1 receptors may contribute to the angiogenic effects of cytokines and growth factors such as IL-1 and VEGF. Furthermore, capsaicin-induced angiogenesis and the reduction in synovial angiogenesis, or synovitis, resulting from the administration of NK_1 receptor antagonists, has previously been interpreted as evidence of an important neurogenic component to synovitis, but could alternatively now be explained by a contribution of HK-1 or EKA/B produced locally by macrophages and other cells within the inflamed synovium [7, 41].

Adrenomedullin

The potential 'handover' of angiogenesis from neuronally derived SP in acute inflammation to inflammatory cell-derived hemokinins in chronic inflammation may be mirrored by CGRP and AM. AM is a 52 amino acid peptide originally isolated from a human pheochromocytoma [53]. It is produced through the cleavage of a 185-amino acid prohormone (pre-proadrenomedullin), which also yields proadrenomedullin N-terminal peptide (PAMP) [54]. *In vitro*, AM has numerous activities on specific cell types, which have been reviewed recently [1]. Of relevance to this review, AM has been shown to promote the proliferation, and migration of cultured endothelial cells. It exerts its action through the cyclic AMP/protein kinase cascade and intracellular calcium mobilisation [55]. This proliferation is inhibited by $AM_{22–52}$ and also by the 'CGRP receptor antagonist', $CGRP_{8–37}$.

Predictably, *in vivo* administration of AM causes vasodilation and a drop in blood pressure. AM was first described as an angiogenic factor in *in vivo* in assays using the chick chorioallantoic membrane [56]. This initial observation has been confirmed and quantified by subsequent studies [57, 58]. As previously mentioned, AM acts preferentially at the CRLR/RAMP2 receptor. However, there are several reports that it is also active at the supposedly specific CRLR/RAMP1 (CGRP) receptor. These studies are based on the sensitivity of the effects of AM to the $CGRP_{8–37}$ fragment, which is relatively specific for the CRLR/RAMP1 receptor [59]. The concentrations at which AM acts at the CGRP receptor are contentious but are likely to be higher than those of CGRP itself. Thus, an analogous situation to the SP/hemokinin example arises in which a chronic inflammatory response would deplete the CGRP-containing nerves but leave CRLR/RAMP1 expression intact. This receptor could then be activated by AM produced by inflammatory cells. AM is known to be produced by inflammatory macrophages during chronic inflammation in the joints of patients with rheumatoid arthritis [60].

Anti-angioangenic peptides, VIP and SOM

It is important not give the impression that all neuropeptides are angiogenic. Two other peptides, VIP and SOM, have been shown to have anti-angiogenic effects on endothelial cells.

Vasoactive intestinal peptide

VIP is a 28-amino acid peptide contained within both the sensory and postganglionic sympathetic nerve fibres. VIP is a member of the glucagon/secretin family

and is a very influential neuropeptide acting as a neurotransmitter or modulator in nearly all tissues [61]. VIP has anti-angiogenic properties both *in vitro* and *in vivo*. It does not have any effect in assays that measure cell proliferation but rather it inhibits the migration of endothelial cells, an equally important step in the formation of new blood vessels [62]. In a model of arthritis, VIP has been shown to be a powerful inhibitor of inflammatory and autoimmune components of the disease [63]. Whether inhibition of angiogenesis contributes to this is not known.

Somatostatin

SOM is also a neuropeptide with wide-ranging functions. Originally characterised as a hypothalamic regulator of growth hormone, SOM also modulates the secretion of multiple pituitary, pancreatic and gastrointestinal hormones such as insulin and glucagon. There are biologically active forms of SOM: SOM14 and SOM28. In mammals these are produced by endoproteolytic processing of the prohormone prosomatostatin, which is, in turn, generated from a 116-amino acid precursor called preprosomatostatin [64]. SOM has been found to be a powerful inhibitor of angiogenesis in several experimental models, for example in the cornea [65]. SOM receptors are widely distributed and there are five subtypes designated sst-1 to sst-5. All the receptor subtypes appear to bind SOM14 and SOM28 with high affinity. Endothelial cells appear to constitutively express sst-1 and sst-3 in the quiescent state but up-regulate sst-2 receptors when activated [66, 67]. SOM has a direct suppressive effect on endothelial cells that is mediated by sst receptors. Sst-2 receptor analogues have received attention as potential therapeutic agents. In addition to a direct effect on vascular endothelial cells, indirect inhibition occurs mediated by SOM's ability to inhibit growth factor secretion such as insulin-like growth factor and VEGF [67].

Conclusion

It is clear that angiogenesis is a complex and tightly controlled process, and that neuropeptide control mechanisms that exist in normal tissue may be different from those in pathology. Initiation of angiogenesis by neuropeptides may 'handover' to its maintenance by related peptides derived from non-neuronal cells, acting on the same, or closely related cell surface receptors. New regulatory pathways involving neuropeptides are being described [68] showing that the nervous system is of importance in vascular remodelling and it is likely that our understanding of the relative importance of various pathways will continue to develop. Anti-angiogenic peptides are already being used in the treatment of retinopathies. Manipulating the neuronal regulation of vascular growth may offer potential therapies for other pathologies.

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The angiogenic drive in chronic inflammation: Hypoxia and the cytokine milieu

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Angiogenesis and underlying mechanisms

In health, angiogenesis, or growth of new blood vessels from pre-existing vasculature, occurs during growth and the female reproductive cycle. It is also a feature of tissue repair following injury and contributes to the pathogenesis of a number of disease states. Angiogenesis arises when hypoxic, diseased or injured tissues secrete pro-angiogenic molecules and is regulated by a complex set of inducers and inhibitors. However, dysregulated angiogenesis contributes to pathological conditions such as chronic gingivitis, diabetic retinopathy, rheumatoid arthritis (RA) and cancer. For the purposes of this brief chapter the example of RA is used to illustrate the clinical correlates of angiogenesis in a pathological setting.

Angiogenesis begins with degradation of the basement membrane of existing vessels by proteolytic enzymes. These include serine proteases of the plasminogen activation system such as urokinase plasminogen activator and matrix metalloproteinases (MMPs). This permits endothelial cells to proliferate and to migrate in a directional manner towards the angiogenic stimulus. A provisional extracellular matrix is laid down, consisting of molecules such as fibrin and fibronectin. Cell adhesion molecules such as members of the αv integrin family (αvβ3 and αvβ5) mediate the interaction of the endothelial cells in the newly formed sprout, as they adhere to one another and to the extracellular matrix. Subsequently, these endothelial sprouts differentiate into mature vessels, which become stabilised and surrounded by pericytes. Finally, the processes of lumen formation, capillary loop formation and stabilisation of the mature vessels occur. The endothelial cells orientate themselves so that the luminal surfaces are aligned creating vessels, which then branch to form the meshwork of capillary loops. Other stimulatory molecules released by both endothelial cells and surrounding cells recruit mesenchymal cells that differentiate into smooth muscle-like pericytes and surround the mature vasculature [1]. A wide range of molecular mediators can potentially promote or inhibit angiogenesis and the overall balance of relevant molecules present at any time determines the strength

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of the angiogenic drive. The wide range of pro-angiogenic molecules includes fibroblast growth factor (FGF), angiopoietins, certain chemokines and cytokines, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), heparin-binding hepatocyte growth factor (HGF) and transforming growth factor-β (TGF-β). One of the best characterised, however, is vascular endothelial growth factor (VEGF) [2], originally described as a vascular permeability factor produced by tumour cells that promoted accumulation of ascites fluid [3].

VEGF (also known as VEGF-A) is the founder member of a family of molecules that includes closely related members such as VEGF-B, VEGF-C and placental growth factor (PlGF). The gene for human VEGF is located on chromosome 6p21.3 and organized into eight exons, separated by seven introns. The resultant mRNA undergoes alternative splicing events to generate several transcripts. The most prevalent form, $VEGF_{165}$, contains 165 amino acids encoded for by exons 1–5, 7 and 8, but lacks the region encoded by exon 6. $VEGF_{121}$ is encoded for by exons 1–5 and 8, with $VEGF_{189}$ and $VEGF_{206}$ also including regions encoded for by exon 6. Less frequently observed variants include $VEGF_{145}$, as well as apparently inhibitory variants that are discussed later. These various VEGF isoforms exhibit different heparin-binding properties, which govern whether the different glycoproteins are secreted or remain cell associated. $VEGF_{165}$ and $VEGF_{121}$ are secreted efficiently from producing cells. This contrasts with $VEGF_{189}$ and $VEGF_{206}$, which remain bound to the cell surface and extracellular matrix, presumed to be because of a highly cationic 24-amino acid sequence encoded by exon 6. The effects of VEGF are mediated *via* binding to two receptor tyrosine kinases, termed Flt-1 or VEGF-R1 and KDR/Flk-1 or VEGF-R2 [4]. VEGF-R1 also binds PlGF and VEGF-B, whereas VEGF-C and -D bind VEGF-R2 and Flt-4 (VEGF-R3). The semaphorin receptors neuropilin (NRP)-1 and NRP-2A form a further subset of VEGF-binding molecules. NRP-1 has been shown to bind $VEGF_{165}$ and thereby enhance VEGF-R2-mediated signal transduction [2].

The primary activity of VEGF is to promote proliferation of endothelial cells *in vitro* and to induce angiogenesis *in vivo* [2, 5]. Many studies have also implicated VEGF in the protection of endothelial cells from apoptosis, acting as a survival factor. This was first described *in vivo* using a murine model of retinopathy of prematurity, in which regression of retinal capillaries was observed in neonatal rats exposed to high oxygen. This turned out to be a consequence of decreased VEGF production by neuroglial cells, leading to endothelial apoptosis [6]. Subsequently, using tetracycline-regulated VEGF expression in a xenografted glioma model, by switching off VEGF production, it was shown that apoptosis of endothelial cells *in vivo* is followed by tumour necrosis [7]. *In vitro*, VEGF was found to protect endothelial cells from cell death induced by serum withdrawal [8]. This important cytoprotective effect of VEGF involves at least in part the induction of inhibitors of apoptosis, namely Bcl-2, XIAP and survivin [9]. In addition to stimulating endothelial cell proliferation and chemotaxis, VEGF induces secretion of interstitial collagenase (MMP-

1) [10] and increases urokinase-type plasminogen activator receptor expression on endothelial cells, thus enhancing the activity of urokinase-type plasminogen activator and presumably endothelial cell invasiveness [11]. VEGF also increases vascular permeability, hence the alternative name of vascular permeability factor [10].

Interaction between hypoxia and cytokines

A feature of VEGF that makes this angiogenic factor particularly relevant in a disease context is its regulation by oxygen tension. *In vitro* studies indicate that VEGF production can be independently up-regulated by pro-inflammatory cytokines and hypoxia but in the context of chronic inflammation *in vivo*, these factors are interdependent (Fig. 1) [12]. Under normal physiological conditions, the microcirculation delivers oxygen to tissues, and the body can adequately cope with changes in oxygen delivery without compromising aerobic respiration. However, the diffusion limit for oxygen is only 100–200 μm, and an increase in tissue mass associated with inflammation leads to an increased distance from the nearest pre-existing blood vessels and thus inefficient oxygenation. The *in vivo* response to the resulting hypoxia is to form new blood vessels [13].

Several distinct molecular mechanisms are thought to be involved in hypoxia-induced up-regulated VEGF expression, at the transcriptional and post-transcriptional levels [14, 15]. Stabilisation of VEGF mRNA occurs *via* binding of various proteins to the VEGF 3'-UTR [16, 17]. Furthermore, VEGF expression is regulated by the 'master regulator' of the adaptive response to alterations in oxygen tension, the hypoxia-inducible factor (HIF), a transcriptional complex containing two (α and β) members of the basic-Helix-Loop-Helix PAS family. HIF molecules bind specifically to hypoxia-responsive elements (HRE) in the promoter or enhancer regions of various genes, which include VEGF, glycolytic enzymes and genes involved in iron metabolism and cell survival. Under normoxic conditions, HIF-α subunits have a short half-life, due to hydroxylation of proline residues by prolyl 4-hydroxylase enzymes (PHD), which require oxygen as a co-substrate [18]. This allows binding of the von Hippel-Lindau ubiquitin ligase complex, which targets HIF-α for proteasomal destruction [19]. Additional oxygen-dependent hydroxylation of asparagines residues within HIF-α regulates recruitment of transcriptional co-activators [20]. The absolute dependence of prolyl and asparaginyl hydroxylation on oxygen means that under conditions of hypoxia, HIF-α accumulates within the nucleus where, upon binding to constitutively expressed HIF-1β and recruitment of the co-activator p300, it recognizes HRE within promoters of target genes such as VEGF, leading to their transcriptional activation. In parallel to the oxygen-dependent pathway, HIF-α is also regulated by receptor-mediated signals, although this pathway is less well understood [21, 22]. These more subtle changes in HIF-α levels and/or transcriptional activation are stimulated by growth factors and cytokines such as tumour

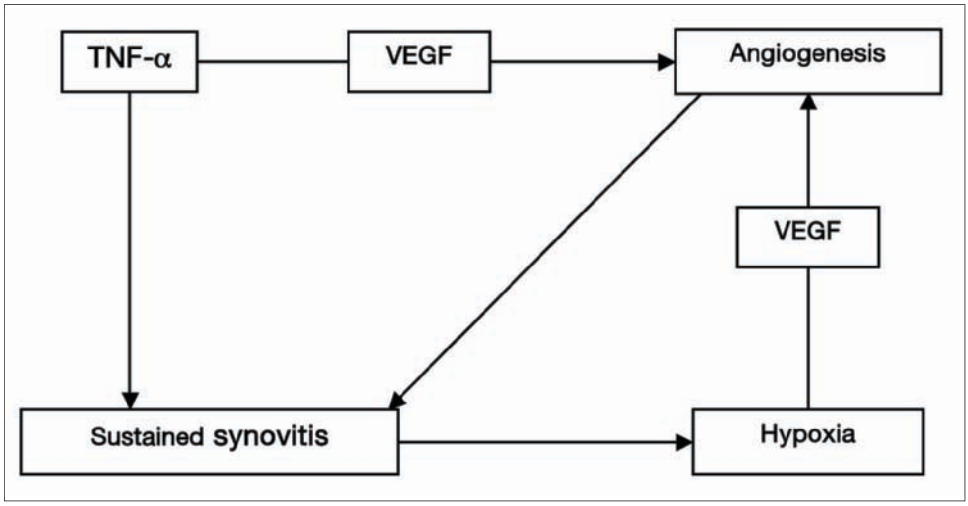

Figure 1
Vascular endothelial growth factor (VEGF) is the most potent growth factor characterised to date with specificity for endothelial cells. In vitro, *VEGF production from synoviocytes can be independently regulated by certain pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α, and by hypoxia. However,* in vivo, *these regulatory factors are interdependent. Metabolically active cells involved in sustained synovitis consume oxygen and promote a hypoxic environment. This environment in turn stimulates VEGF production and formation of new blood vessels in an unsuccessful attempt at restoring oxygen homeostasis.*

necrosis factor (TNF)-α and interleukin (IL)-1. For example, TNF-α was shown to up-regulate HIF-1α protein [23–26]. Other studies have shown TNF-α-mediated up-regulation of HIF-1α DNA-binding activity [22, 27]. The up-regulation of HIF-1α is thought to involve stabilisation of protein, possibly through prevention of degradation of ubiquitinated HIFs by PHDs [26, 28]. Thus, cytokines that regulate VEGF expression through activation of inflammatory signalling pathways such as NF-κB [29] may also contribute to angiogenesis by induction of VEGF through a HIF-dependent mechanism.

Clinical correlates of angiogenesis

RA is characterised by chronic inflammation of synovial joints with synovial proliferation and infiltration by blood-derived cells, in particular, memory T cells, macrophages and plasma cells, all of which show signs of activation [30–32]. Angiogenesis in the synovial membrane of RA patients is considered by many investigators to be an important early step in pathogenesis of RA and in the perpetuation of disease [33,

34]. Histologically, luxuriant vasculature is a prominent feature of RA synovitis [35] and the disease activity of a given joint is correlated with the synovial vascularisation [36]. Angiogenesis can be evident on microscopic examination of synovial biopsies from the earliest stages of disease evolution and is observed as a fine network of vessels over the rheumatoid synovium at arthroscopic inspection of RA joints. Angiogenesis is integral to the development of inflammatory pannus and without it, leucocyte ingress could not occur. Furthermore, formation of new blood vessels permits a supply of nutrients and oxygen to the augmented inflammatory cell mass and so contributes to the perpetuation of synovitis. In the chronic phase of disease, capillaries and post capillary venules are particularly evident in the synovial sub-lining region. In histological sections, mononuclear and polymorphonuclear leucocytes can sometimes be found in close apposition to vascular endothelium, probably in the process of margination and adhesion prior to migration into the inflamed tissue. The synovial tissue becomes markedly hyperplastic and locally invasive at the interface of cartilage and bone with progressive destruction of these tissues in the majority of cases. This invasive tissue is referred to as 'pannus', comprising mainly lining cells with the appearance of proliferating mesenchymal cells with very little sub-lining lymphocytic infiltration. Cytokine-induced degradative enzymes, most notably the MMPs, are the major mediators of bone and cartilage destruction.

It is now generally accepted that the synovial vasculature is central to maintaining and promoting RA, in that the expansion of synovial tissue necessitates a compensatory increase in the number of synovial blood vessels. The number of synovial blood vessels correlates with synovial cell hyperplasia, mononuclear cell infiltration and indices of joint tenderness [37]. Endothelial cells lining blood vessels within RA synovium have been shown to express cell cycle-associated antigens, including Ki67, as well as integrin $\alpha v\beta 3$, which is associated with vascular proliferation [38, 39].

Many pro- and anti-angiogenic factors have been reported to be expressed in RA synovium [40, 41]. Members of the FGF family (FGF-1 and FGF-2) have been detected in human RA synovial tissue [42]. Similarly, PDGF, a potent mitogen for many cell types including fibroblasts and smooth muscle cells, is expressed in RA synovium [43]. HGF has been found at significant levels in RA synovial fluids, with levels higher in RA compared to osteoarthritis (OA) [44]. The potential role of TGF-β during the course of RA, and in synovial angiogenesis, is unclear. TGF-β was shown to induce VEGF expression in human synovial fibroblasts [45]. Indeed, TGF-β is by far the most powerful inducer of VEGF secretion by human synovial fibroblasts, when compared with other cytokines associated with the pathogenesis of RA, such as IL-1 or PDGF. Thus, it seems likely that in RA, TGF-β exerts its angiogenic effects predominantly through the induction of VEGF secretion by fibroblasts. VEGF levels are elevated in the serum and synovial fluids of RA patients, relative to either patients with OA or normal controls [46–48], and correlate with levels of C-reactive protein, a marker of inflammation and disease activity [49]. There is a significant correlation between serum VEGF at presentation with early RA and the

magnitude of radiological deterioration, calculated using hand and feet radiographs taken at initial presentation and at 1-year follow-up, suggesting that high serum VEGF levels at an early stage of disease are associated with the increased joint damage [50]. In addition to VEGF, receptors (VEGF-R1, VEGF-R2 and NRP-1) are also expressed in RA synovium [48, 51]. The same receptors are also expressed in human OA and cartilage [52–54], with a report describing predominant expression of $VEGF_{121}$ and $VEGF_{189}$ isoforms [55]. Similarly, VEGF is present in OA synovial fluids, albeit at lower levels than in RA [46].

Despite active angiogenesis, the RA joint is hypoxic [56]. In the seminal work of Lund-Olesen and colleagues mean synovial fluid pO_2 in RA knee joints was reported to be as low as 27 mmHg compared to 43 mmHg in OA and 63 mmHg in traumatic effusions in otherwise "healthy" controls [57]. Although an increase in local blood flow has been reported, this is unlikely to be sufficient to compensate for the increased requirement for oxygen and nutrients. Using a murine arthritis model, onset of disease has been shown to be associated with a reduction in synovial oxygen tension [58]. HIF-1α has been demonstrated to be expressed in the lining and sub-lining areas of RA joints, predominantly in areas with high levels of macrophage infiltration, supporting the hypoxia-induced pathway of VEGF up-regulation [59, 60]. In an animal model of arthritis, HIF-1α was shown to be associated with areas of hypoxia in inflamed joints [61]. Expression of HIF-2α has also been reported [62]. One of the consequences of synovial hypoxia is up-regulation of VEGF release and promotion of a pro-angiogenic state [49, 63]. Collectively, these data suggest that angiogenesis and hypoxia promote and maintain the inflammatory and destructive drive in RA synovium.

Although the role of the prototypical VEGF molecule, VEGF-A, has been extensively studied in the context of RA, the roles of other related molecules are less well understood. PlGF has been detected in the synovial fluid and plasma of patients with RA [64]. The contribution of another member of the VEGF family, VEGF-B, has been examined using knockout mice. The severity of arthritis induced using either adjuvant or collagen was found to be reduced in mice lacking VEGF-B, and this effect was associated with decreased synovial vessel density [65]. Interestingly, increased VEGF-B mRNA was detectable in the synovial tissue of arthritic mice mainly as the more diffusible $VEGF\text{-}B_{186}$ isoform, rather than $VEGF\text{-}B_{167}$. VEGF-C and -D are thought to predominantly regulate lymphangiogenesis. The role of lymphatic vessels in RA is not well studied. A recent report has shown co-expression of vascular markers (such as CD31) with the lymphatic endothelial hyaluronan receptor (LYVE-1) in areas of chronic inflammatory cell infiltrates, both in OA but more particularly in RA [66]. This is in accordance with the description of VEGF-C localization in RA synovium (in the synovial lining layer and stromal cells), together with mRNA for VEGF-C receptors VEGF-R2 and -R3. Little VEGF-D was expressed in RA synovium [67, 68]. More recently, inhibitory splice variants of VEGF-A have been described. In particular, $VEGF\text{-}A_{165}b$, resulting from the differential splicing

from the end of exon 7 into the 3' UTR of VEGF mRNA to yield a 165-amino acid peptide with an alternative C-terminal 6-amino acid sequence has been described as inhibiting responses induced by VEGF-A_{165}, i.e. proliferation and migration of endothelial cells. It is of interest that expression of VEGF-A_{165}b is reduced in patients with renal tumours compared to healthy controls, suggesting that other angiogenesis-dependent diseases, such as RA, may also exhibit similar patterns of differential expression of stimulatory and inhibitory VEGF isoforms [14, 69].

The involvement of EGF in human cancer has also motivated interest in the EGF/EGF receptor family in other diseases associated with angiogenesis [70]. EGF has been detected in RA synovial fluids and in synovial membranes, with expression increased relative to patients with OA [71, 72]. EGF and related molecules such as neuregulins and TGF-α bind the ErbB family of receptor tyrosine kinases, comprising ErbB-1 (also known as EGF-R, HER), -2, -3 and -4. EGF receptors are activated by ligand-induced dimerisation. Although ErbB-2 does not bind any of the EGF receptor ligands with high affinity, this receptor nonetheless can dimerise with other members of the EGF receptor family. A report in the literature showed that ErbB-2 is expressed in RA, but not OA, synovium. EGF stimulated the phosphorylation of ErbB-2 in RA synovial fibroblasts in RA, and this was inhibited by herceptin, a monoclonal antibody against ErbB-2, which is approved for use in breast cancer [73].

Angiopoietins are postulated to play a role in vessel stabilisation. The angiopoietin family comprises several structurally related proteins, the best characterised being angiopoietin-1 and -2. Angiopoietin-1 activates its receptor Tie (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains)-2, unlike angiopoietin-2, which appears to act as an antagonist/partial agonist. It has been hypothesised that angiopoietin-1 promotes differentiation and stabilisation of endothelial cells in the newly formed vascular networks, whereas angiopoietin-2 inhibits the action of angiopoietin-1 and hence works to initiate neovascularisation. Expression of both angiopoietin-1 and angiopoietin-2 in RA synovial tissue has been described [74, 75], together with receptors Tie-1 and -2 [76, 77]. Psoriatic arthritis may present with a range of clinical patterns, some of which are associated with joint destruction and may resemble RA clinically. Interestingly, expression of angiopoietin-2 and VEGF is reported to be higher in the synovia of patients with psoriatic arthritis, relative to RA, whereas angiopoietin-1 levels were more comparable. Furthermore, distinct patterns of vascular morphology are reported to occur in psoriatic arthritis and RA. Blood vessels in psoriatic synovium are highly tortuous in appearance at arthroscopy, in contrast to the straight and branching vessels seen in RA, suggesting that the balance between angiopoietin-1, angiopoietin-2 and VEGF may affect vessel growth and maturation in arthritic synovium [78].

Angiogenesis may not be the only process contributing to new vessel formation in RA synovium. Angiogenesis describes the extension of existing vessels into new areas, but in the embryo, formation of the primitive vasculature occurs *via*

vasculogenesis through the *de novo* differentiation of progenitor cells from mesoderm-derived precursor cells termed haemangioblasts [79]. Endothelial and haematopoietic cells are known to be derived from the same precursor cells because of the presence of common markers, including VEGF-R2, angiopoietin receptors, CD31 and CD34 [80, 81]. The differentiated haemangioblasts form clusters of endothelial cells (or blood islands), which multiply and fuse to give rise to the dorsal aorta, cardinal vein and yolk sac. Intra-embryonic vascular development follows yolk sac vascularisation. Vasculogenesis has also been suggested to occur postnatally, and bone marrow-derived cells expressing CD34 and VEGF-R2 have been described. Endothelial precursor cells expressing CD34, VEGF-R2 and CD133 are also reported to be expressed in RA and OA synovial tissue [82].

Summary

Many angiogenic factors are expressed in chronic inflammatory conditions such as RA, as well as indices of hypoxia such as HIF transcription factors. Despite the increased vascularity associated with RA synovitis, the RA joint is hypoxic. Repetitive cycles of hypoxia and reoxygenation together with oxidants produced by phagocytic cells promote chronic oxidative stress within the microenvironment of the joint, leading to generation of reactive oxygen species with the potential to cause tissue damage. Changes in cellular oxygenation regulate intracellular concentrations of the transcription factor HIF-1α that activates a gene program permissive to perpetuation of synovitis.

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Dendritic cells and angiogenesis

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Introduction

Dendritic cells (DC) are professional antigen-presenting cells with a unique ability in inducing T and B cell response as well as immune tolerance [1, 2]. DC reside in an immature state in peripheral tissues where they exert a sentinel function for incoming antigens. Upon microbial contact and stimulation by inflammatory cytokines, DC uptake antigens, undergo a process of maturation, and traffic *via* the afferent lymphatics into the T cell area of the draining lymph node to initiate immune responses [3, 4].

DC are a heterogeneous population in terms of origin, morphology, phenotype, and functions [5]. In humans, three main types of DC can be identified: (1) Langerhans cells, resident in epithelia; (2) interstitial DC, localized in peripheral tissues (including derma); and (3) plasmacytoid DC, preferentially localized in secondary lymphoid tissues. Two main circulating human blood DC subsets can be distinguished on the basis of differentially expressed surface markers: Lin^- $CD11c^+$ MHC-DR^+ myeloid DC, and Lin^- $CD11c^-$ BDCA-2^+ and BDCA-4^+ plasmacytoid DC. Plasmacytoid DC were previously known as interferon (IFN)-producing cells since they are the main producer of type I IFNs [6]. The two subsets express a different panel of pattern recognition receptors and therefore respond to different pathogen-associated molecular patterns [5, 7, 8].

DC play a pivotal role in the onset and regulation of adaptive immune responses. DC control Th1/Th2 polarization and the state of tolerance to self antigens and allergens [2, 9]. Immature DC may induce regulatory T cells, thus promoting tolerance, whereas mature DC stimulate effector T cells, supporting immunity [2, 10]. Although the primary biological function of DC is the initiation of specific immune responses, DC share with other phagocytes the ability to regulate inflammatory responses through their ability to release cytokines and chemokines, kill bacteria and regulate angiogenesis (Fig. 1) [11–15].

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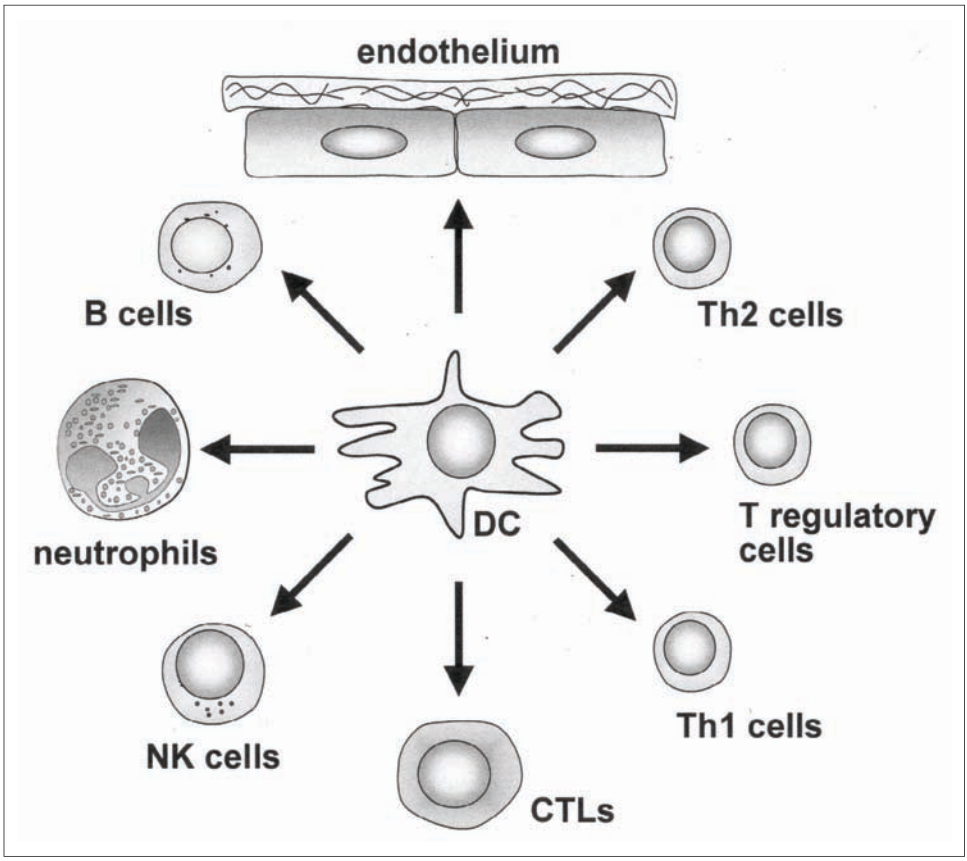

Figure 1
Regulatory role of dendritic cells (DC) in inflammation and immunity. DC, on the basis of the activation signals that they encounter, are capable of delivering different signals that regulate the activation of several effector cells.

DC produce pro- and anti-angiogenic mediators

DC have an extraordinary capacity to produce bioactive molecules that act in an autocrine and/or paracrine manner. The cytokine milieu that characterizes the local microenvironment affects the phenotype and function of antigen-presenting cells present at the pathological site. For instance, in the presence of pro-inflammatory agonists, such LPS or interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α), classic activated DC (CA-DC) acquire a mature phenotype associated with the ability to promote Th1 responses and to release inflammatory chemokines. Con-

versely, the simultaneous presence of pro- and anti-inflammatory signals promotes an "alternative" state of activation (alternatively activated DC, AA-DC) defined by a distinct cytokine profile and specific functions [16, 17]. Even though the contribution of DC to angiogenesis has so far obtained limited attention, experimental evidences indicate that DC may express both pro- and anti-angiogenic mediators *in vitro* and *in vivo*.

Myeloid CA-DC release several cytokines endowed with direct or indirect pro-angiogenic properties, including TNF-α, IL-6, and transforming growth factor-β (TGF-β) [18, 19]. On the other hand, DC express IL-12. This cytokine, a key regulator of immune processes (reviewed in [20]), is endowed with anti-angiogenic properties [20]. IL-12 production depends upon subtype and state of maturation of DC and is finely regulated by the cytokine milieu, being potently stimulated by IFN-γ [20]. For instance, CA-DC release high amounts of IL-12, whereas IL-12 expression is completely abolished in AA-DC. Myeloid CA-DC constitutively express also IL-18, a cytokine that shares biological activity with IL-12, including anti-angiogenic properties [21].

DC are an important source of chemokines. Chemokines are a large family of chemotactic proteins distributed in four subfamilies [22]. Chemokines of the CXC subfamily characterized by the presence of an ELR amino acid motif promote angiogenesis, the most representative member being CXCL8. On the other hand, ELR^- CXC chemokines (CXCL10, CXCL9, CXCL4, and CXCL14) inhibit angiogenesis [23]. Again, myeloid CA-DC express both pro-angiogenic (mainly CXCL8, as well as CXCL1, CXCL2, CXCL3, CXCL5, and CXCL7) and anti-angiogenic (CXCL9, CXCL10) chemokines [12, 18, 24–26]. Myeloid DC also produce CCL21, a chemokine of the CC subfamily that possesses angiostatic properties [27, 28].

Even though modifications in the balance between pro- and anti-angiogenic mediators may decide the angiogenic behaviour of CA-DC, we have observed that both human CA-DC and immature DC do not exert a significant angiogenic activity *in vitro* and *in vivo* [13]. In contrast, blood-purified myeloid DC and monocyte-derived DC alternatively matured in the presence of anti-inflammatory molecules [i.e. calcitriol, prostaglandin E_2 (PGE_2), or IL-10] secrete high amounts of the prototypic angiogenic growth factor vascular endothelial growth factor (VEGF) [13]. Accordingly, AA-DC display a potent angiogenic activity *in vivo* that is hampered by neutralizing anti-VEGF antibodies or by the tyrosine-kinase VEGF receptor inhibitor SU5416 [13]. Altogether, these results indicate that AA-DC induce angiogenesis *in vivo* mainly through the production of VEGF. As mentioned above, the alternative activation of myeloid DC dramatically impairs the production of anti-angiogenic IL-12. In contrast, IL-10 and PGE_2 up-regulate the production of the natural angiogenesis inhibitor thrombospondin-1 (TSP-1) in DC [29]. Therefore, like CA-DC, also AA-DC may express both pro- and anti-angiogenic cytokines. However, the *in vivo* pro-angiogenic activity of AA-DC reported in our study suggests that, at least under defined experimental conditions, the balance of these two

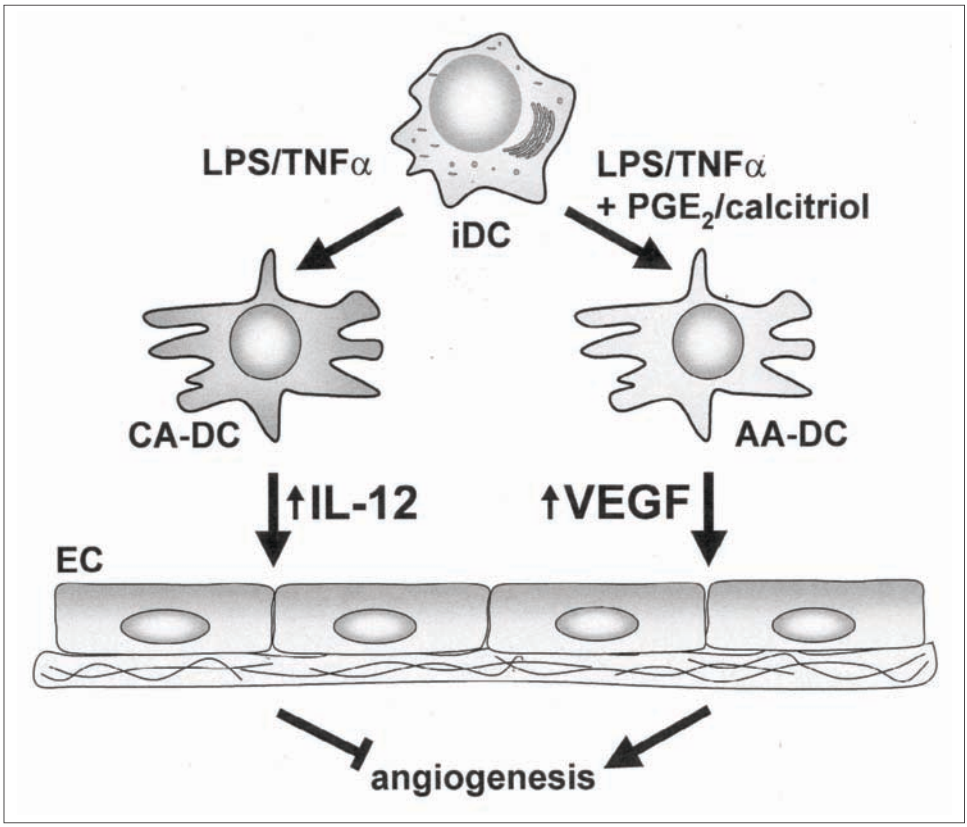

Figure 2
Different angiogenic behaviour of classically activated (CA-DC) and alternatively activated (AA-DC) DC. Immature DC (iDC) activated by pro-inflammatory plus anti-inflammatory signals mature to AA-DC and express high levels of vascular endothelial growth factor (VEGF), thus exerting a potent angiogenic activity in vivo. *In contrast, CA-DC express the anti-angiogenic IL-12, with a possible negative impact on the neovascularisation process.*

activities favours angiogenesis (Fig. 2). Since the resolution phase of inflammation is characterised by the presence of anti-inflammatory signals, we propose that AA-DC may contribute to this process by promoting neovascularisation *via* an increase in VEGF production paralleled by IL-12 down-regulation. Alternatively polarising signals are also produced under different pathological conditions, including chronic inflammation and cancer. Thus, AA-DC may represent a source of angiogenic factors in all these settings. Recent observations have confirmed the ability of monocyte-derived DC to secrete VEGF when matured in the presence of TNF-α/LPS and PGE_2 [30]. Also, $CD11c^+$ DC have been shown to mediate the VEGF-dependent

vascular growth in reactive lymph nodes [15]. Furthermore, *in vivo* expression of VEGF by myeloid DC has been observed in skin biopsies from patients with leprosy [31, 32] and in murine DC [14].

Plasmacytoid DC are a rare subset of DC and the main source of IFN-α (reviewed in [33]). IFN-α inhibits endothelial cell motility and stimulate the production of ELR^- CXC chemokines [34]. Accordingly, activated plasmacytoid DC express the anti-angiogenic chemokines CXCL9 and CXCL10 [35]. However, as observed for myeloid DC, activated plasmacytoid DC also express the pro-angiogenic CXCL8 [35] and tumour-derived plasmacytoid DC may promote angiogenesis through the release of TNF-α and CXCL8 [36]. However, at variance with AA-DC, we failed to induce VEGF up-regulation in plasmacytoid DC activated by influenza virus in the presence of calcitriol or PGE_2 [13].

In conclusion, depending upon the activation status and the cytokine milieu, DC may express both pro- and anti-angiogenic mediators, thus suggesting that DC may exert a different impact on the neovascularisation process under different physiopathological conditions.

DC respond to pro- and anti-angiogenic mediators

Both positive and negative mediators of the angiogenic process can affect the biology of DC. Even though these mediators are expressed by a variety of cell types, they are frequently produced by the DC themselves, thus acting as autocrine factors.

Pro-angiogenic molecules

VEGF represents the most studied example of angiogenic modulator active on DC. The expression of the different tyrosine kinase VEGF receptors (VEGFRs) on human DC precursors and DC has been extensively investigated. Human $CD34^+$ cells express VEGFR-1 [37–39], whereas the presence of VEGFR-2 on these cells is controversial [38, 40, 41]. $CD34^+$ cell-derived human DC express VEGFR-1 but not VEGFR-2 [38]. Monocytes, a precursor of myeloid DC, also express VEGFR-1 but not VEGFR-2 [39, 42–44]. Furthermore, the expression of VEGFR-3 has been described in corneal DC but not skin DC [45, 46]. Interestingly, neuropilin-1, a VEGF co-receptor, was found to be identical to BDCA-4, a specific marker used to identify and purify blood plasmacytoid DC [47]. The role of neuropilin-1 in the biology of this DC subset is still unclear. Neuropilin-1 expression is also induced during *in vitro* differentiation of monocytes into DC [30, 48]. In keeping with the expression of signalling VEGFRs on these cells, VEGF was shown to recruit $CD34^+$ progenitor cells, monocytes, Langerhans cells, and corneal DC [44, 46, 49–51].

VEGF inhibits the differentiation of haemopoietic progenitor cells into functional DC. Gabrilovich and colleagues described that supernatants from several breast and colon adenocarcinoma cell lines affect the ability of human haematopoietic progenitor cells to become functional DC *in vitro* [38]. Indeed, DC obtained in the presence of tumour supernatants demonstrate features of immature cells, VEGF being the main factor responsible for this effect [52–54]. Tumour-derived VEGF also induces a significant decrease of the number and function of spleen and lymph nodes DC [55]. Finally, in a mouse model of asthma, VEGF over-expression in the airway increases the number of AA-DC [56].

Various angiogenic activators, distinct from VEGF, can act on DC, including TGF-β and hepatocyte growth factor (HGF) [57]. TGF-β plays a crucial role in the development of Langerhans cells (reviewed in [58]). Indeed, TGF-β1-deficient mice lack Langerhans cells [59]. Moreover, TGF-β1 inhibits DC maturation, promoting the generation of tolerogenic DC [60]. Similarly, HGF has been shown to down-regulate the antigen-presenting capacity of DC and to exert a protective role in a mouse model of allergic airway inflammation [61].

DC produce osteopontin (OPN) and endothelin-1 (ET-1), two molecules promoting angiogenesis. DC differentiation, maturation, and survival are influenced by OPN and ET-1 in an autocrine and/or paracrine manner [62, 63]. The extracellular matrix protein OPN plays important functions in inflammation and vascular remodelling [64]. In mice, OPN is required for the migration of DC/Langerhans cells from the skin to the draining lymph nodes. OPN-deficient mice show a defect in the ability to mount an effective contact hypersensitivity response [65]. Recombinant OPN activates monocyte-derived DC and polarise them into Th1-promoting DC *in vitro* [66]. Yet, the role of OPN in Th1 responses is controversial [67, 68]. Very importantly, OPN has been shown to exert a pivotal role in IFN-α production by plasmacytoid DC [69].

Anti-angiogenic molecules

There are only few reports about the effect of angiogenesis inhibitors on DC biology, these inhibitors being TSP-1 and CXCL4.

TSP-1 is expressed by monocyte-derived DC and its production is regulated by soluble mediators and maturation signals. TSP-1 is constitutively secreted by immature DC and its secretion is up-regulated after maturation [29]. Anti-inflammatory molecules provide a positive signal for TSP-1 secretion. TSP-1 secretion in monocyte-derived DC is potently stimulated also by extracellular adenosine triphosphate (ATP) and ATP-induced TSP-1 has an anti-proliferative effect on $CD4^+$ T lymphocytes [70]. TSP-1 can act as an autocrine negative regulator of human DC activation, impairing their ability to release cytokines. In addition, exogenous TSP-1 was reported to down-modulate DC maturation [71].

CXCL4 is an ELR$^-$ CXC chemokine released by activated platelets. CXCL4 can influence differentiation and function of monocyte-derived DC [72]. Moreover, monocytes cultured in the presence of IL-4 and CXCL4 differentiate in antigen-presenting cells with unique properties that set them apart from conventional DC [73]. These cells have a specific phenotype (e.g. they express BDCA-3), induce only moderate cytokine responses, and promote T lymphocytes proliferation and lytic NK activity.

DC/endothelial cell plasticity

The close developmental relationship between haematopoietic and endothelial lineages has suggested the hypothesis that both cell types arise from a common mesodermal progenitor, the haemangioblast [74]. Actually, experiments performed with blast cell colonies generated from embryonic stem cells have demonstrated that haematopoietic and endothelial precursors within the blast colonies develop from the same cell [75].

Recently, several lines of experimental evidence have shown that DC and endothelial cells are closely related. DC respond to angiogenic modulators and, in turn, endothelial cells may act as antigen-presenting cells [76]. There is evidence of a phenotypic overlap between monocyte-derived DC and microvascular endothelium [43, 77]. Tumour-derived VEGF recruits CD34$^+$ progenitors and induces their proliferation. In culturing conditions used to stimulate the differentiation of DC, angiopoietins present in tumour-conditioned media can skew CD34$^+$ cell differentiation towards endothelial cell differentiation at the expense of DC development [50]. A new population of CD11c$^+$ leucocytes exhibiting both endothelial and DC features has been recently uncovered in murine carcinomas co-expressing VEGF and β-defensins, a class of anti-microbial peptides [78]. DC precursors chemoattracted by β-defensins are transformed by VEGF into endothelial-like cells able to assemble vascular structures *in vivo*. The analysis of human ovarian carcinomas led then to the identification of a novel population of cells with leucocyte/endothelial-like phenotype named vascular leucocytes (VLC) [79]. It has been hypothesized that VLC originate from leucocytes recruited by the tumour at a low stage of differentiation. These cells will then dually differentiate towards DC and endothelium in the tumour microenvironment. On the other hand, the "endothelial-like switch" of DC into VLC can be observed *in vitro* by culturing CD34$^-$ CD11c$^+$ DC in media conditioned by tumour cells expressing high levels of VEGF [80]. The phenomenon of "DC/endothelial cell plasticity" is somehow reminiscent of the vasculogenic mimicry observed in tumours [81]. Cells from aggressive melanoma have a high plastic phenotype, can express endothelium-associated genes, and form vasculogenic-like networks in three-dimensional culture. Human aggressive tumours show the presence of primitive networks anastomosed to endothelium-lined vasculature, possibly

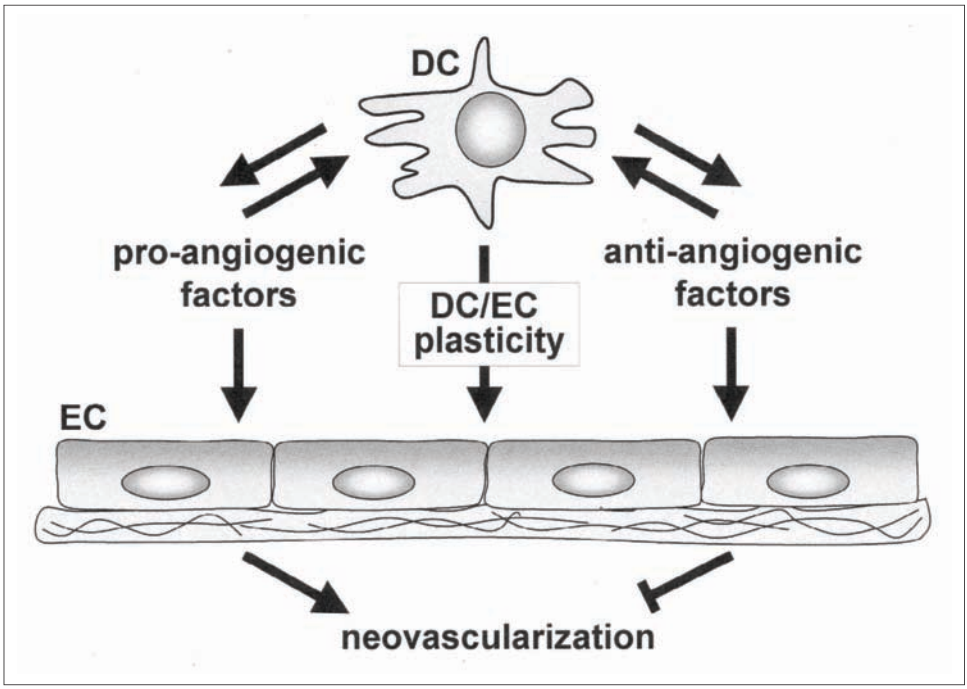

Figure 3
Impact of DC on neovascularisation. DC may modulate neovascularisation by acting on existing vessels through the release of pro- and anti-angiogenic factors. Also, DC may contribute to vasculogenesis by direct trans-differentiation into endothelial-like cells (EC). In turn, pro- and anti-angiogenic mediators may affect DC functions.

providing a paracirculation in the tumour area. Vascular mimicry has been observed also in non-melanoma tumour types, including carcinomas of the breast, prostate, ovary, and lung.

In conclusion, DC may contribute to the formation of new vessels in two ways: (1) stimulating angiogenesis from existing vessels through the release of pro-angiogenic factors; and (2) contributing to vasculogenesis by direct differentiation into endothelial-like cells (Fig. 3).

Concluding remarks

DC are professional antigen-presenting cells situated at the interface between innate and adaptive immunity. In the past few years, DC have been reported to play a regulatory function in several processes, including vascular growth. Depending

upon the activation status and the cytokine milieu, DC may express both pro- and anti-angiogenic mediators, thus suggesting that DC may exert a different impact on the neovascularisation process in different physio-pathological conditions. Moreover, DC are targets of pro- and anti-angiogenic factors. The interplay between DC and angiogenic modulators results in modifications of DC biology as well as in DC participation to angiogenesis. Surprisingly, DC seem to play a role in angiogenesis not only through their insurmountable ability to secrete cytokines, but also through trans-differentiation into endothelial cells. Clearly, the comprehensive understanding of DC involvement in angiogenesis will need further exploration. A better knowledge of molecules and mechanisms involved in the formation of new vessels in chronic inflammation and in cancer will provide novel therapeutic approaches.

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The lymphocyte in inflammatory angiogenesis

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Introduction

The ability of immune cells to recognise foreign pathogens, while simultaneously maintaining tolerance towards proteins produced by the body's own cells, forms the basis of mammalian immunity. At the heart of the immune system are the lymphocytes, which orchestrate the adaptive immune response through clonal expansion upon recognition of a specific antigen. The plasticity of the immune system allows exquisite control of the body's defences. However, the adaptive immune system can also be directed towards host proteins ('self antigens'). The reasons for this failure in immunity are varied, and include a genetic basis or evasion of the host immune response by viruses. Nevertheless, the consequences – autoimmune diseases such as rheumatoid arthritis (RA) – are frequently associated with inflammation, immune cell dysfunction and changes in the vasculature.

In particular, new blood vessel formation (termed 'angiogenesis') is a recurrent theme in the context of inflammatory autoimmune disorders such as RA [1–3]. The cascade of events perpetrated by macrophages, dendritic cells and lymphocytes leads to generation of inflammatory cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-1, which can in turn induce expression by inflammatory and immune cells of modulators of angiogenesis, such as vascular endothelial growth factor (VEGF). Furthermore, the proliferation associated with RA or cancer results in an increased requirement for oxygen and nutrients, and hence a need for further blood vessel formation, which apart from improving oxygenation would also bring in more inflammatory cells and molecules to the site of inflammation. Thus, cells of the immune system are intricately linked with the vasculature, through molecular cross-talk involving cytokines and growth factors – soluble and cell-associated – producing an exquisitely regulated interplay of inflammation, immunity and angiogenesis. The reciprocal relationships between inflammatory processes, immune cells and angiogenesis form the focus of this chapter.

Angiogenesis in Inflammation: Mechanisms and Clinical Correlates, edited by Michael P. Seed and David A. Walsh

Lymphocyte function in chronic inflammatory diseases

The vascular system is essential to the activation of an effective immune response. Integration of antigen presentation, amplification of lymphocytes and the generation of mediators of humoral and cellular immunity occur in the peripheral lymphoid organs, primarily lymph nodes and spleen. T cells circulate between non-lymphoid tissues and the lymph nodes, entering through the afferent lymphatics and across the high endothelial venules, and exiting *via* efferent lymphatics. This continuous lymphocyte trafficking enables the antigen-sensitive cells to be exposed to their specific antigen, prompting clonal expansion. Blood vessels allow recruitment of the activated lymphocytes to specific sites, which is promoted by vasodilation and an increase in vessel density through angiogenesis. Thus, angiogenesis will allow the ingress of cells and molecules to the site of inflammation, maintaining the immune response and potentially promoting further blood vessel growth through lymphocyte-mediated expression of angiogenic factors such as VEGF.

RA is the prototypic autoimmune disease, although the self antigen(s) that prompt the body to mount a response is not clear as yet. The autoimmune nature of RA is underlined by the association with genetic elements encoded within the human leucocyte antigen (HLA)-DR region. More than 80% of Caucasian RA patients express HLA-DR1 or HLA-DR4 subtypes. The association between specific HLA class II alleles and the development of RA supports the widely held view that T lymphocytes are pivotal to RA development [4]. The primary site of inflammation in RA is the synovial membrane lining of the closed spaces of articular joints, which increases greatly in mass, and becomes infiltrated by blood-derived cells of lymphohaematopoietic origin, including T cells, B cells and macrophages. The lymphocytes infiltrating the synovium are predominantly $CD4^+$ T cells, with high expression of memory CD45R0 antigens and activation markers such as HLA-DR and CD69. These T cells have been proposed to express a hyporesponsive phenotype, which has been suggested to result from prolonged exposure to cytokines such as TNF-α. An *in vitro* study, in which antigen-activated T cells (mimicking those which might be present during the early antigen-driven phases of RA) were exposed to TNF-α for prolonged periods of time, showed that such T cells exhibited suppression of cytokine production (in particular IL-2) and reduced antigen-induced proliferation [5]. TNF-α-treated T cells also required exposure to higher peptide/MHC complexes for longer periods to commit to IL-2 production. This was mediated through uncoupling of T cell receptor (TCR) signalling pathways, such that assembly of the TCR/CD3 complex was impaired, due to down-regulation of TCRζ chain expression, leading to attenuated downstream signal transduction pathways [6, 7]. In RA synovium, pro-inflammatory cytokines such as TNF-α are continuously expressed, implying that this induction of T cell hyporesponsiveness may indeed occur *in vivo* [8, 9].

An interesting aspect of the immune response in RA is the formation of tertiary (ectopic) lymphoid structures, underlining the complexity of T cell:B cell

interactions in this disease [10, 11]. These lymphoid microstructures have been observed in the synovial membrane, which is the primary site of inflammation in RA. The cells infiltrating synovium can form several types of distinct aggregates, with unique cytokine profiles. So-called 'diffuse' areas contain a haphazard array of T cells, B cells and blood vessels in the sub-intimal synovial layer. Alternatively, the lymphocytes form aggregates, which may be distributed deep in the synovium, away from blood vessels, and/or in apposition to blood vessels, sometimes with development of classical germinal centres resembling those seen in secondary lymphoid organs. One of the original studies to describe this phenomenon reported that more than 60% of synovial tissue was diffuse in appearance. The remainder displayed lymphoid organ-like appearance, with B cells clustering in follicle-like arrays in apposition to T cells. This second sub-group was approximately equally divided into T cell:B cell aggregates without follicular dendritic cell networks and into aggregates resembling classical germinal centres [12]. The presence of germinal centres has been reported to be associated with transcription of lymphotoxin β and CXCL13 [13], although other studies have shown that the B cell chemokine CXCL13 is also expressed in the absence of fully formed follicles in RA synovitis [14]. Although thought by some to differentiate between sub-groups of RA patients and hence potentially between different pathogenic processes, these distinct histomorphological features can actually be seen in different areas of synovium from the same RA patient [15].

An early study of the graft *versus* host reaction demonstrated that injection of lymphocytes into histoincompatible hosts resulted in formation of an intricate network of blood vessels [16]. Thus, in addition to fostering the breakdown of tolerance, lymphocytes – at the heart of the immune response and the inflammatory aggregates in RA synovium – may also contribute to the angiogenesis, which is now recognised as fundamental to the development of autoimmune diseases such as RA.

Hypoxia-mediated regulation of lymphocyte function

Inflammation in the context of an immune reaction requires the lymphocyte to cross the vascular endothelial lining of blood vessels to the underlying tissue, where the cell encounters inflammatory stimuli and antigen-presenting cells. Such extravasation means that lymphocytes leave the relatively high oxygen concentrations of the circulation and traverse deep into areas that may be quite distant from the closest capillaries. Efficient delivery of oxygen to cells depends on the distance from the nearest blood vessels not exceeding 200 μm, which is the diffusion limit for oxygen. However, in an inflammatory milieu, this limit is often exceeded, as new vessel formation due to angiogenesis fails to keep pace with alterations in cellular proliferation and metabolism. This leads to regions of hypoxia and hypoperfusion, which are

characteristics of the tumour microenvironment, as well as chronic inflammatory states such as RA.

The RA synovium can be perceived as a tertiary lymphoid tissue, as a consequence of the lymphoid neogenesis described above, and would therefore be expected to be hypoxic. Moreover, in RA, lymphocytes in the inflamed synovium are likely to be exposed to lower levels of oxygen, and/or to fluctuations in oxygen tension, since accumulation of fluid temporarily obliterates capillary flow in the synovium, compounding the reduced perfusion still further. RA synovial tissue has indeed been shown in many studies to be extremely hypoxic. As in the case of tumour growth, the mass of the hyperplastic RA pannus may result in local hypoxia, thus driving the need for a compensatory neovascularisation, to increase the supply of nutrients and oxygen [17, 18]. Up-regulation in RA synovium of hypoxia-sensitive glycolytic enzymes such as glucose-6-phosphate isomerase has been described [19], and this phenomenon of hypoxia within the RA joint is supported by observations of decreased synovial fluid oxygen tensions [17, 20]. We have measured synovial oxygen tension in RA patients using a highly sensitive gold microelectrode, and observed that synovial tissue in RA patients was more hypoxic (< 6% O_2) than non-inflamed synovium in patients without RA (approximately 10% O_2). Furthermore, we observed that areas of RA synovium that were invading underlying tissue (such as tendon) were even more hypoxic (3% O_2) [21]. Similarly in an animal model of arthritis, onset of disease was associated with a marked reduction in synovial oxygen tensions [22]. Several studies have demonstrated that the oxygen consumption of the RA synovium is elevated, possibly due to the increased proliferative activity of synovial cells, and that glucose is oxidized *via* an anaerobic, rather than aerobic, pathway [17, 23, 24]. It is thus apparent that lymphocytes are exposed to different oxygen tensions, depending on their spatial orientation, ranging from peripheral arterial blood (generally 10–15% O_2), to approximately 8–10% O_2 in well-vascularised tissue such as muscle, and lower values in secondary lymphoid organs such as spleen [25, 26].

The consequences of hypoxia on immune cell function have not been extensively studied. At this point, it is worth pointing out that there is little agreement about what constitutes 'hypoxia'. As discussed above, oxygen tensions can vary even under physiological conditions, ranging from arterial blood levels to much lower tissue levels. Furthermore, many studies are carried out in comparison to atmospheric oxygen levels, namely 20–21% O_2. Thus, some authors' definition of 'hypoxia' may actually be more analogous to physiological 'normoxia'. In general, levels of O_2 below 5% are considered to be 'hypoxia'. In the late 1990s, Naldini and co-workers [27, 28] reported that peripheral blood mononuclear cells stimulated with phytohaemagglutinin (PHA) released significantly more lymphocyte mediators – IL-2, IL-4 and interferon (IFN)-γ – under hypoxic conditions (2% O_2), relative to normoxic controls. Release of IL-10 decreased in both resting and PHA-stimulated cells. Similar findings of increased IL-2, IL-4 and IFN-γ release by mitogen-

stimulated peripheral blood mononuclear cells exposed to 5% O_2 were reported by another group [29]. A more recent study showed that exposure of spleen cells to hypoxia lead to development of fewer $CD8^+$ T cells (relative to $CD4^+$), but with a greater cytotoxic activity. TCR-activated cells exposed to hypoxia (2.5% O_2) were also found to release more VEGF, which is hypoxia regulated, but less IL-2 and IFN-γ than under normoxic conditions [25]. Furthermore, it has been reported that activation-induced cell death (AICD) of human T cells triggered by CD3 was reduced by hypoxia (< 5% O_2), although T cells incubated in the absence of CD3 showed reduced viability in hypoxia [30].

Taken together, these results suggest that under hypoxic conditions in the absence of TCR/CD3 ligation, T cells may release more cytokines but may also be more prone to apoptosis, but survival of antigen-stimulated T cells may be favoured, thereby allowing preferential survival of specific T cells in an inflammatory microenvironment such as RA synovium. This is also supported by the hyporesponsive phenotype of T cells observed in RA [9].

The HIF pathway and angiogenesis

A highly efficient system existsto respond to changes in oxygen tension. The ability of cells to adapt to periods of hypoxia is important for their survival, and it is crucial that cells respond to hypoxia by expressing a variety of proteins to adapt to the stress of low oxygen tension [31]. A principal regulator of this adaptive response is the transcriptional complex termed hypoxia-inducible factor (HIF). Functional HIF is a heterodimer composed of two basic helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) proteins, i.e. HIF-1α and the aryl hydrocarbon nuclear translocator (ARNT) known as HIF-1β [32]. At least three forms of HIF-α have been described (HIF-1α, -2α and -3α), with the ubiquitously expressed HIF-1α and HIF-2α thought to function as activators of gene transcription *via* binding to a hypoxia response element (HRE). Transactivation of HIF target genes involves subunit dimerisation, formation of a complex with co-activators, and binding to HRE located in HIF target genes [33, 34]. Two transactivating domains have been identified in the C-terminal part of the HIF-1α protein, i.e. an N-terminal domain (N-TAD) and more downstream C-terminal domain (C-TAD). The C-TAD in particular has been shown to interact with co-activators such as p300/CBP to activate transcription. Further upstream of the transactivating domains, a contiguous bHLH/PAS domain creates a functional interface for subunit dimerisation and binding to HRE [34]. Regulation of HIF activity is achieved primarily by post-translational modifications, which affect its half-life and/or transcriptional activity, and include hydroxylation, ubiquitination, acetylation, S-nitrosation and phosphorylation. HIF-α sub-units contain an oxygen-dependent degradation domain, which is the target of HIF-α prolyl hydroxylases (PHD) [35, 36]. In humans, three HIF-specific PHD (PHD1,

PHD2, and PHD3; originally designated EGLN-2, -1 and -3, due to their sequence homology with the HIF-1 PHD of *Caenorhabditis elegans* EGF-9) have been identified. These enzymes require 2-oxoglutarate, iron and O_2 for their activity, and hydroxylate HIF-α in the defined oxygen-dependent degradation (ODD) domain, which overlaps with the N-TAD [33]. Prolyl hydroxylation (on Pro402 and Pro564 in human HIF-1α) allows HIF-α to interact with von Hippel Lindau (VHL) tumour suppressor, which is part of the ubiquitin E3 ligase complex. Following polyubiquitination, HIF-α-subunits are subjected to proteolytic destruction by the 26S proteasome. The absolute requirement of PHD for oxygen means that during hypoxia, the decline in oxygen levels abolishes PHD activity, preventing hydroxylation and hence degradation of HIF-α, allowing HIF-α to accumulate, dimerise with HIF-β and activate transcription of genes containing HRE [37, 38]. Interestingly, PHD2 and PHD3 are themselves HIF target genes, which would accelerate HIF-α degradation after re-oxygenation [39–42]. HRE elements have been found in approximately 1–2% of human genes, and over 60 genes are now believed to be regulated by HIFs, including VEGF [31]. A further pathway that regulates HIF-α involves the oxygen sensor FIH-1 (factor inhibiting HIF-1), a dioxygenase that hydroxylates asparagine residues in the C-TAD (Asn803 in human HIF-1α and Asn851 in human HIF-2α) in an oxygen-dependent manner. This prevents interaction of HIF-α sub-units that have escaped proteasomal degradation with transcriptional co-activators such as p300/CBP [43], and therefore inhibits transactivation. FIH is thought to be able to limit transcriptional effects of HIF even at low oxygen levels, under which conditions PHD enzymes are no longer active [44].

As discussed previously, the RA synovium is thought to be a relatively hypoxic microenvironment, and it would be anticipated that HIF-α levels would be up-regulated. Indeed, HIF-1α, HIF-2α and target genes such as VEGF are expressed in RA synovium [45–47], as well as in experimental arthritis models [48], suggesting that synovial hypoxia leads to up-regulation of HIF in the joint, accumulation of VEGF and induction of synovial angiogenesis. We have observed that HIF-1α and HIF-2α are not only expressed in human RA joint synovium, but also in RA synovium invading tendons of the hand. HIF-2α appears to be consistently expressed in the synovial intimal lining layer, as well as within areas of cellular infiltrates deeper within the synovium. In contrast, HIF-1α is expressed in particular by endothelial cells lining blood vessels [21].

The observation that HIFs, in particular HIF-2α, are expressed within cellular infiltrates in RA synovium, histomorphologically resembling the T cell:B cell clusters described by Weyand and colleagues [11], supports the concept that in RA lymphocytes play key roles in the response to varying oxygen tensions. Another study aimed at assessing the role of HIFs specifically in T cell function, and demonstrated that in RA synovium HIF-1α expression co-localised with CD3 expression [30]. To specifically evaluate the role of the HIF system in lymphocyte differentiation and function, RAG-2 (recombination-activating gene 2)-deficient mice, which have

no mature B and T lymphocytes due to the inability to initiate VDJ recombination, were employed. Blastocysts from RAG-2-deficient mice were injected with pluripotent embryonic stem cells with a homozygous deletion of the gene encoding for HIF-1α, to generate somatic chimeras with mature B and T cells, all of which derived from the injected embryonic stem cells, thereby overcoming the embryonic lethality of HIF-1α-deficient mice. These animals displaying HIF-$1\alpha^{-/-}$ T and B lymphocytes showed grossly normal proportions of T cell subsets (CD4 and CD8) in thymus, spleen, lymph nodes and peripheral blood. Interestingly, the B cell phenotype was altered, with appearance of peritoneal B1-like lymphocytes showing high expression of B220 (CD45) receptor-associated protein tyrosine phosphatase. The HIF-$1\alpha^{-/-}$ chimeric mice also exhibited an autoimmune phenotype, with increased serum levels of IgM and IgG anti-double-stranded DNA antibodies and deposition of immunoglobulins in the kidneys [49]. As discussed above, AICD of human T cells triggered by CD3 was shown to be reduced by hypoxia. This somewhat unexpected finding was shown to be paralleled by HIF-1α protein accumulation, suggesting that HIF-1α plays a role in the regulation of AICD of T cells under hypoxic conditions. DNA array analysis revealed that while hypoxia up-regulated many of the anticipated targets, such as glucose transporters (GLUT3) and VEGF, ligation of CD3/TCR did not further up-regulate these genes. In contrast, adrenomedullin was increased modestly by hypoxia, but the combination of TCR/CD3 stimulation and hypoxia enhanced expression of this gene still further, suggesting that hypoxia promotes survival of activated T cells *via* adrenomedullin [30]. The increased HIF-1α accumulation was found not be mediated by increased protein stability, but rather through increased protein synthesis, possibly through activation of the pathway involving phosphatidylinositol 3-kinase and the mammalian target of rapamycin (mTOR) system, since rapamycin was shown to inhibit expression of both HIF-1α and its target genes (such as VEGF) in TCR/CD3-stimulated T cells [50]. Conditional knockout of VHL (using the *Cre* recombinase approach under the control of the lck promoter to achieve thymic deletion of VHL and hence constitutive expression of HIF-1α protein) has been shown to result in a profound suppressive effect on signalling downstream of TCR ligation. TCR-mediated influx of Ca^{2+} was markedly diminished, an effect which was restored in double-knockout mice lacking thymic expression of both VHL and HIF-1α genes, supporting the hypothesis that HIF-1α, as opposed to the other HIF-α sub-types, was involved. Interestingly, TCR proximal signalling (activation of phospholiase C γ) was unaffected in $VHL^{-/-}$ thymocytes. The HIF-1α-mediated effect was instead proposed to be mediated by HIF-1α-dependent expression of the Ca^{2+} pump SERCA2, leading to increased Ca^{2+} removal from the cytoplasm [51].

What might be the consequence of lymphocytes being exposed to a hypoxic milieu in terms of angiogenesis? As briefly mentioned earlier, injection of lymphocytes into histoincompatible hosts resulted in formation of an intricate network of blood vessels, suggesting that lymphocytes can express angiogenic factors during

the course of an inflammatory response [16, 52, 53]. The classic hypoxia-regulated gene is VEGF, and hypoxia-inducible VEGF mRNA expression has been shown in $CD3^+$ peripheral blood T lymphocytes, Jurkat cells and in $CD4^+$ and $CD8^+$ T cell subsets [54]. We have shown in several studies that hypoxia is a potent stimulus for VEGF induction in RA synovial membrane cell cultures, which contain lymphocytes as well as macrophages and fibroblasts [55, 56]. In RA synovium, we have also demonstrated that VEGF expression appears to closely resemble that of HIF-1α and HIF-2α [21]. Forced overexpression of HIF-1α in thymocytes, using the lck-directed conditional inactivation of VHL, resulted in the overexpression of HIF-1α angiogenic targets, such as VEGF. Although the size of the thymus was reduced in $VHL^{-/-}$ mice, the organs were highly vascularised, a consequence which might be expected from increased VEGF levels [57]. TCR-activated lymphocytes exposed to hypoxia were found to release more VEGF [25]. In another study, T cell lines were generated from lymph node cells and shown to express VEGF in response to IL-2 and specific antigen challenge. Culture of activated T cells in the absence of oxygen lead to further up-regulation of VEGF [58]. In addition to expressing angiogenic mediators, it is thought that T cells can also signal to other cells to induce angiogenesis, particularly *via* ligation of CD40, which is expressed by most antigen-presenting cells and endothelial cells. CD40 ligand (CD154) in turn is expressed on activated T cells. Ligation of CD40 was shown to induce VEGF expression by endothelial cells and monocytes [59, 60]. Culture of RA fibroblasts with CD40L-expressing cells or activated T cells was also shown to induce VEGF [61].

Thus, it could be hypothesised that in RA, cytokines such as TNF-α could lead to uncoupling of proximal TCR signals [6], whereas concomitant hypoxia could further reduce T cell responses by down-regulating any remaining TCR signalling events. Moreover, hypoxia-mediated induction of HIF target genes such as VEGF would promote further angiogenesis, bringing in antigen-specific lymphocytes to the site if inflammation.

Conclusions

Angiogenesis is important for the entry of cells and molecules to the site of inflammation, maintaining the immune response and potentially promoting further blood vessel growth. The combination of cytokines and hypoxia in an inflammatory micro-environment such as RA synovium is likely to alter the T cell phenotype, leading to alteration in signalling pathways and expression of angiogenic factors such as VEGF. The apparent links between the immunosuppressive activity of rapamycin on T cell signalling pathways with actions on hypoxia-regulated gene expression are of interest, and certainly there is evidence implicating mTOR as an upstream activator of the HIF pathway, which might explain the anti-angiogenic effects of rapamycin

[62, 63]. The chemokine CXCL12:CXCR4 axis also links lymphocyte function with angiogenesis. CXCL12, or stromal cell-derived factor 1 (SDF-1) is a chemotactic and angiogenic factor involved in the homing of stem and progenitor cells to areas of tissue damage and inflammation. HIF-1-driven expression of this chemokine in fibroblasts and endothelial cells is likely to both promote recruitment of T and B lymphocytes to RA synovium, and to increase the homing of CXCR-4$^+$ bone marrow-derived endothelial progenitor cells to form new blood vessels [64, 65]. Thus, lymphocytes and endothelial cells are part of an elaborate cellular network, involving cross-talk of cytokines, growth factors and ligand:counter-ligand interactions, promoting and maintaining inflammatory disorders such as RA.

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The fibroblast and myofibroblast in inflammatory angiogenesis

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Wound repair: Granulation tissue formation and angiogenesis

Normal wound healing includes a number of overlapping phases. After injury, there is an early inflammatory step characterised by haemorrhage and clotting. At this time, the wound has a provisional serum-derived extracellular matrix, which serves to seal the wound temporarily and allows the invasion of cells that carry out the repair process. In the next phase, consisting of granulation tissue development, fibroblasts invade the wound and commence replacing the provisional matrix with a more mature wound matrix. The fibroblasts present during the early granulation tissue phase resemble immature fibroblasts with a highly synthetic appearance. However, as the granulation tissue phase proceeds, fibroblasts start showing a new phenotype with prominent contractile structures represented by microfilament bundles or stress fibres; these structures express contractile proteins typical of smooth muscle cells, particularly of vascular smooth muscle cells, such as α-smooth muscle actin [1]. Recently, it has been shown that α-smooth muscle actin is largely responsible for force production by the myofibroblast both *in vitro* and *in vivo*. Myofibroblast differentiation is a complex process, regulated by at least one cytokine [transforming growth factor (TGF)-β1] [2], an extracellular matrix component (fibronectin ED-A) [3] as well as the presence of mechanical tension [4] (for review, see [5]). Lastly, in the resolution phase of healing, there is considerable loss of cellularity essentially through apoptosis of several cell types including myofibroblasts [6]. The signals for this cell death are unknown. Conversely, inappropriate delay of apoptosis, and thus increased survival of myofibroblasts during the healing process, may be a factor that leads to excessive scarring. This latter proposition, however, lacks conclusive evidence to date. In hypertrophic scars, α-smooth muscle actin-positive myofibroblasts are commonly present in nodules [7]. Recently, differential responses to apoptotic inductors were observed between normal skin wound and hypertrophic scar myofibroblasts, confirming the hypothesis of defects in apoptosis

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and growth during pathological scar formation impeding myofibroblast disappearance [8]. During the last 40 years the concept of granulation tissue contraction has been clarified in many aspects. Further work on the biology of the myofibroblast will definitively contribute to the understanding and the control of normal and pathological connective tissue remodelling.

The myofibroblast differentiation

Myofibroblasts exhibit a phenotype intermediate between that of fibroblasts and smooth muscle cells (Fig. 1). In general, myofibroblasts are α-smooth muscle actin positive but are desmin negative except in some pathological situations [1]. Myofibroblasts may also pass through an early phase where they have prominent microfilament bundles but have not yet acquired α-smooth muscle actin expression (proto-myofibroblast, Fig. 1). It has been hypothesised that myofibroblast differentiation depends on exposure to growth factors and other environmental factors such as mechanical stress and extracellular matrix molecules [4, 5]. Myofibroblasts also exhibit gap junctions that interconnect them and have specialised connections to the extracellular matrix, termed the fibronexus, which assures the continuity between intracellular microfilament proteins and the extracellular matrix.

The most important regulator of myofibroblast phenotype is TGF-β1 [2]. Granulocyte macrophage colony-stimulating factor (GM-CSF) has also been suggested to increase the number of myofibroblasts in subcutaneous tissue. However, it is likely that since GM-CSF shows no direct effect on fibroblasts *in vitro*, its *in vivo* effects are mediated by macrophage recruitment and activation; these cells in turn produce TGF-β thus initiating myofibroblast differentiation. Other growth factors that are mitogenic for fibroblasts appear to have no direct effect on α-smooth muscle actin induction, e.g. platelet-derived growth factor (PDGF) and connective tissue growth factor, which stimulate fibroblast proliferation but do not appear to induce myofibroblast differentiation (for review, see [9]).

As mentioned above, myofibroblasts have important interactions with the extracellular matrix through fibronexus attachments. Early in most tissue repair phenomena there is a provisional extracellular matrix composed largely of fibrin and fibronectin to which cells adhere. It has been shown that the ED-A sequence of cellular fibronectin is required for induction of the myofibroblast phenotype [3]. Recently, new possible mediators of fibroblast to myofibroblast differentiation have been reported such as thrombin, acting through the protease-activated receptor-1, and endothelin-1 [10, 11].

A number of factors that inhibit myofibroblast differentiation have also been identified. In cultured fibroblasts, interferon-γ decreases α-smooth muscle actin expression as well as fibroblast proliferation. Other studies have shown that interferon-γ can inhibit myofibroblast expression *in vivo* in both Dupuytren's contrac-

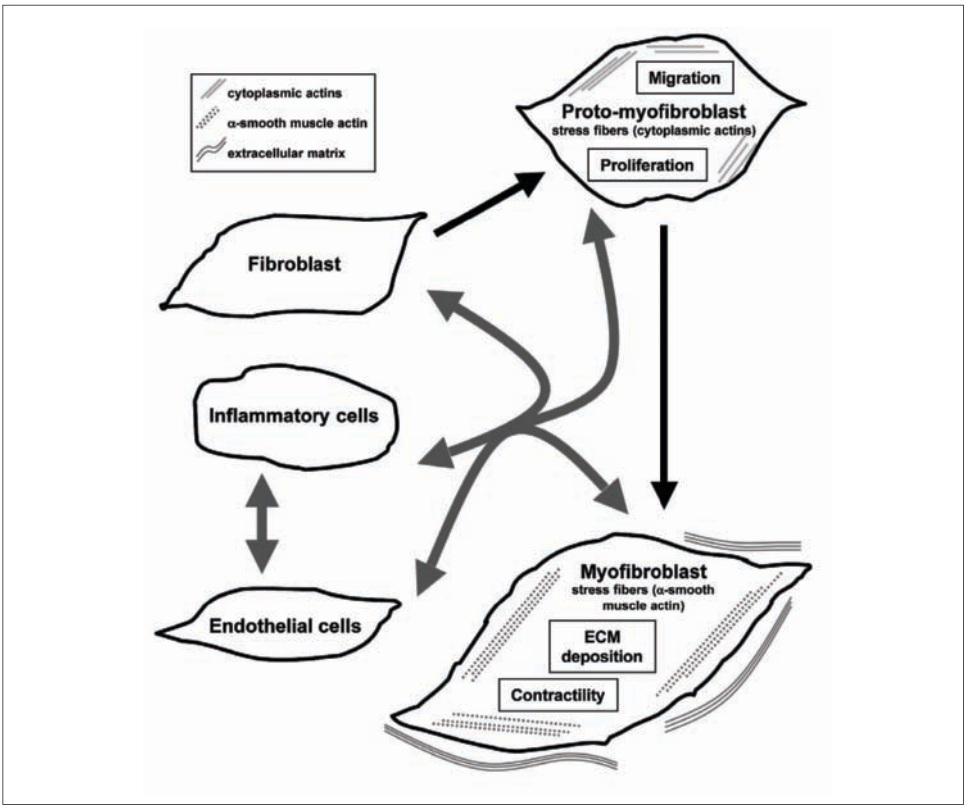

Figure 1
Schematic illustration showing the evolution of the (myo)fibroblast phenotype.
The myofibroblastic modulation of fibroblastic cells begins with the appearance of the proto-myofibroblast, whose stress fibres contain only β- and γ-cytoplasmic actins and evolves, but not necessarily always, into the appearance of the differentiated myofibroblast, the most common variant of this cell, with stress fibres containing α-smooth muscle actin. Inflammatory cells and endothelial cells secrete mediators able to modify (myo)fibroblast phenotype.

ture and hypertrophic scars. In addition, the pregnancy hormone relaxin has been shown to down-regulate collagen production and contractility in fibroblasts, suggesting that relaxin may be able to inhibit the myofibroblast phenotype [12, 13]. Lastly, while it is usual to observe about 10% of α-smooth muscle actin-positive cells (myofibroblasts) in a monolayer culture derived from the dermis, only few cells are positive when this heterogeneous "fibroblast" population is seeded within a collagen matrix, suggesting a negative selection, and/or an inhibition of myofibroblast differentiation, depending on the surrounding collagen matrix. Interestingly, this

effect seems tissue specific since part of the "fibroblast" population derived from gingiva continue to express α-smooth muscle actin when embedded within a 3-D collagen matrix [14].

Myofibroblasts, angiogenic factors, and the stroma reaction

Angiogenesis represents the main mechanism of tissue vascularisation, followed by vasculogenesis and arteriogenesis. As mentioned above, angiogenesis is a formation of blood vessels from preexisting ones. Moreover, angiogenesis may be exerted through distinct mechanisms: sprouting, intussusception and cooption. Vasculogenesis is the formation of blood vessels by recruitment of endothelial precursor cells (EPC) named angioblasts and present in the bone marrow (for review, see [15]). In arteriogenesis, mural cells, i.e. smooth muscle cells and pericytes are recruited along the nascent vasculature and contribute to muscularisation of the vessel. All these mechanisms are controlled by growth factors and other secreted proteins such as proteases and extracellular matrix components or by cell-cell interactions. Myofibroblasts (as well as fibroblasts) present in wounds are able to secrete some of these growth factors (Tab. 1); they are also involved in the synthesis of extracellular matrix components and of proteins implicated in extracellular matrix remodelling (Tab. 2).

Among these secreted proteins, we find all the growth factors implicated in developmental angiogenesis, i.e. vascular endothelial growth factor (VEGF)-A, angiopoietin (Ang) 1 and 2, fibroblast growth factor (FGF)-2, PDGF, TGF-β, and growth factors such as keratinocyte growth factor (KGF), epidermal growth factor (EGF) and hepatocyte growth factor (HGF). The angiogenic activity of these last factors is, at least partly, mediated by an increase in VEGF-A production and secretion by endothelial and stroma cells [16]. Another group of angiogenic/vasculogenic factors secreted by activated fibroblasts is composed of cytokines, such as interleukin (IL)-1β, or chemokines, such as stromal derived factor (SDF)-1 implicated in inflammation. During cancer and rheumatoid arthritis (RA), these critical factors can interact with endothelial cells and induce angiogenesis; they can also recruit inflammatory cells secreting conventional angiogenic factors such as VEGF-A or FGF-2 [17]. Depending on the pathological situation, IL-18 exerts opposite effects on angiogenesis; it may be a potent inducer of angiogenesis in RA but shows inhibitory properties in tumour [18, 19]. This may be explained by the presence of different IL-18 receptors on endothelial cells or by an indirect cytokine activity; IL-18 induces synthesis of pro-angiogenic factors such as VEGF-A and chemokines containing the Glu-Leu-Arg (ELR) motif (e.g. IL-8 and the epithelial neutrophil activating protein-78) in RA and of angiogenic inhibitors such as thrombospondin 1 or interferon-γ in tumour. Activated fibroblasts synthesise trombospondin1 and 2, two large proteins with anti-angiogenic activity. These molecules inhibit endothelial cell proliferation, migration and survival, and therefore angiogenesis.

Table 1 - Soluble factors secreted by activated fibroblasts and implicated in vascularisation.

Factor	Role in
VEGF-A	Vasculogenesis (EPC recruitment), angiogenesis.
VEGF-C	Angiogenesis
Ang1	Angiogenesis (vessel stabilisation), vasculogenesis (EPC recruitment)
Ang2	Angiogenesis (Ang1 antagonist)
FGF-2	Vasculogenesis, angiogenesis, arteriogenesis
KGF	Angiogenesis (microvascular endothelial cells)
TGF-β	Angiogenesis, arteriogenesis (stimulates ECM production)
PDGF	Angiogenesis, arteriogenesis (recruits smooth muscle cells)
EGF	Angiogenesis (increase VEGF-A production)
HGF	Angiogenesis (partly by increasing VEGF-A production)
TNF-α	Angiogenesis, arteriogenesis
SCF	Vasculogenesis (EPC recruitment)
TF	Angiogenesis
IGF-1	Vasculogenesis, angiogenesis
Angiogenin	Angiogenesis
Substance P	Angiogenesis
Prolactin	Angiogenesis inhibitor (inhibits FGF-2 and VEGF-A)
IL-1β	Inflammatory angiogenesis
IL-6	Inflammatory angiogenesis
IL-8	Inflammatory angiogenesis
IL-13	Inflammatory angiogenesis (partly by increasing VEGF-A production)
IL-15	Inflammatory angiogenesis
IL-18	Angiogenesis inhibitor in tumour (inhibit EC migration and FGF-2); angiogenesis activator in rheumatoid arthritis
GM-CSF	Vasculogenesis, angiogenesis, arteriogenesis
G-CSF	Vasculogenesis, Angiogenesis
SDF-1	Vasculogenesis (EPC recruitment)
MCP-1	Angiogenesis, arteriogenesis
Tsp1	Angiogenesis inhibitor (inhibits EC migration, growth and survival)
Tsp2	Angiogenesis inhibitor (inhibits EC migration, growth and survival)

Ang, angiopoietin; EC, endothelial cell; ECM, extracellular matrix; EGF, epidermal growth factor; EPC, endothelial precursor cell; FGF, fibroblast growth factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte macrophage-colony stimulating factor; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; Il, interleukin; KGF, keratinocyte growth factor; MCP, monocyte chemoattractant protein; PDGF, platelet-derived growth factor; SCF, stem cell factor; SDF, stromal derived factor; TF, tissue factor; TGF, transforming growth factor; TNF, tumour necrosis factor; Tsp, thrombospondin; VEGF, vascular endothelial growth factor.

Table 2 - Extracellular matrix effectors synthesised by activated fibroblasts and implicated in vascularisation.

Factor	Role in
Collagen type I	Angiogenesis
Collagen type III	Angiogenesis
Collagen type V	Angiogenesis
Fibronectin	Angiogenesis
MMP-1	Angiogenesis
MMP-2	Angiogenesis
MMP-3	Angiogenesis
MMP-11	Angiogenesis
Urokinase	Angiogenesis
Heparanase	Angiogenesis
Elastase	Angiogenesis
TIMP	Angiogenesis inhibitor
PAI-1	Angiogenesis inhibitor
Syndecan-1	Angiogenesis

MMP, matrix metalloproteinase; PAI, plasminogen activator inhibitor; TIMP, tissue inhibitor of matrix metalloproteinases.

VEGF-A represents the main regulator of angiogenesis under normal and pathological conditions. It was originally characterised as a vascular permeability factor. Further work has shown that VEGF-A is implicated in endothelial cell proliferation, migration, and survival *in vitro*. VEGF-A is a heparin-binding homodimeric glycoprotein occurring in at least five isoforms of 121, 145, 165, 189 and 201 amino acids due to alternative splicing of a single gene. VEGF-A 165 is, generally, the major isoform and VEGF-A 121 is a freely diffusible molecule. The principal VEGF-A inducer is hypoxia; under low oxygen pressure the transcription factor hypoxia inducible factor (HIF)-1α is up-regulated and activates VEGF-A transcription. Numerous growth factors such as EGF, PDGF, KGF, FGF-2, TGF-β and insulin-like growth factor (IGF)-1, or inflammatory cytokines such as IL-1α, IL-6 or IL-13, secreted by fibroblasts, induce expression of VEGF-A. Secreted VEGF-A binds to two tyrosine kinase receptors, VEGFR1 and VEGFR2 essentially found on endothelial and bone marrow-derived cells such as the monocyte. VEGFR2 activation induces endothelial cell proliferation, survival and migration, and VEGFR1 activation induces protease and growth factor production by endothelial cells. At least in embryonic angiogenesis, VEGFR1 is a decoy receptor for VEGFR2 and regulates angiogenesis activated by VEGFR2. Two co-receptors, neuropilin 1 and 2 (NP1 and NP2), with no known signalling activity, were recently characterised. NP1

binds VEGF-A (except the 121 isoform) and presents the growth factor to VEGFR2 [20]. Beside its angiogenic activity, VEGF-A induces EPC recruitment from bone marrow, a process mediated by VEGFR2 and abolished by neutralizing antibodies against VEGFR2. Moreover, VEGF-A induces recruitment of VEGFR1 positive haematopoietic stem and progenitor cells in the vasculature and may participate in inflammatory angiogenesis [21].

Recently, fibroblasts present in tumours (activated) were found to induce vasculogenesis. Orimo et al. [22] have injected breast carcinoma cells mixed with activated or non-activated fibroblasts into mice. Those injected with activated fibroblasts developed bigger tumours with high levels of angiogenesis. Moreover, a large number of EPC, positive for Sca1 and CD31, were found within tumour vasculature and inside blood. VEGF-A, stem cell factor (SCF) (c-Kit ligand), Ang1, SDF-1 and MMP-9 have been described as potent inducers of EPC recruitment. Among these inducers, SDF-1 was found up-regulated in activated fibroblasts. SDF-1 belongs to the chemokine family, composed of CC, CXC and CX3C chemokines, based on the presence or absence of an amino acid (X) between a pair of cysteine residues near the N-terminal extremity. All the CC, such as monocyte chemoattractant protein (MCP)-1, and the CXC chemokines containing the ELR motif are angiogenic. The CXC chemokines missing the ELR motif, such as PF-4 or IP-10, are angiostatic, with the exception of SDF-1. In human, two splice variants, α and β, derived from a single gene have been identified. SDF-1 expression is increased by hypoxia-induced HIF-1α and by NF-κB and down-regulated by TGF-β and steroids. SDF-1's unique known receptor, CXCR4, is a G protein-coupled transmembrane protein found almost exclusively in stem cells such as haematopoietic, endothelial, liver oval or tumour stem cells. SDF-1 binding to CXCR4 regulates locomotion, chemotaxis, adhesion and secretion [23]. Altogether, SDF-1 is secreted by activated fibroblasts, binds to CXCR4 present at the cell surface of haematopoietic and endothelial stem cells and recruits them to the site of injury where they participate in vasculogenesis and inflammation.

In addition to the secretion of growth factors, cytokines and chemokines, activated fibroblasts participate in inflammatory angiogenesis by secreting and organizing an abundant extracellular matrix that traps angiogenic growth factors (Tab. 2). Activated fibroblasts secret extracellular matrix components, such as type I, III, and V collagens or fibronectin, and enzymes implicated in extracellular matrix remodelling. Among these enzymes are metalloproteinases (MMP-1, 2, 3 and 11), heparanase, urokinase and plasmin, all implicated in angiogenesis. Moreover, activated fibroblasts express tissue inhibitors of MMP (TIMPs) and plasminogen activator inhibitor (PAI)-1, able to inhibit, respectively, MMPs and urokinase. Recently, expression of syndecan-1 at the plasma membrane of fibroblasts was shown to stimulate angiogenesis [24]. Syndecan-1 belongs to the syndecan family of type 1 transmembrane heparan sulphate proteoglycans. The N-terminal extracellular domain of the core protein is followed by an ectodomain containing Ser-Gly consen-

sus sequences for heparan sulphate or, in some cases, chondroitin sulphate attachment. The C-terminal intracellular domain is composed of two highly conserved and one variable domains. These domains can interact with several molecules such as tubulin, calmodulin-associated serine/threonine kinase (CASK), phosphatidylinositol 4,5-biphosphate (PIP2) and protein kinase (PK)Cα. Recently, it was shown that HGF binding to extracellular domain of syndecan promotes activation of PI3-kinase and mitogen-activated protein kinase (MAPK) pathways by Met. Moreover, following FGF-2 binding to its receptor, activated protein phosphatase 1/2A dephosphorylates syndecan-4, inducing PIP2 binding, which in turn promotes syndecan-4 multimerisation and PKCα activation. Many angiogenic factors (EGF, FGF-2, PDGF, TGF-β, HGF and VEGF-A), chemokines or cytokines (IL-8, MCP-1 and GM-CSF) or cell adhesion molecules (L-, P- and E-selectin or platelet endothelial cell adhesion molecule-1) are able to bind heparan sulphate. These interactions have implicated syndecan in inflammatory cell maturation, activation and adhesion to the endothelium. Syndecan-1 is found in normal epithelial cells and, transiently, in mesenchymal cells during embryonic development [25]. Aberrant syndecan-1 expression has been described in 70% of activated fibroblasts in infiltrating breast carcinoma and correlated with tumour high vessel density and high average vessel area. This was confirmed by *in vivo* experiments, where human mammary carcinoma cells mixed with fibroblasts expressing or not expressing syndecan-1 were injected to immunodeficient mice. Tumour volumes and weights were higher if fibroblasts express syndecan-1. Vessel density and average vessel area were increased, suggesting the presence of larger vessels in these tumours and confirming a role of syndecan-1 in angiogenesis when expressed at the fibroblast cell surface. Syndecan-1 presents FGF-2 or other heparan sulphate binding factors such as VEGF-A to their respective receptor at the endothelial cell surface [24].

Specificities of the liver sinusoid microenvironment

The liver parenchyma is divided into functional units called lobules. The lobules are polygonal, generally hexagonal; each is 1–2 mm in diameter and is composed of a labyrinth of interconnected hepatocyte plates separated by endothelium-lined sinusoids. Each lobule is crossed by the centrolobular vein. The hepatocyte plates radiate out from the centrolobular vein to the perimeter of the lobule; the portal triads (portal vein, hepatic artery, and bile ductule), and the surrounding connective tissue are typically found at the angles of the polygon. Sinusoids are lined by endothelial cells and Kupffer cells. Kupffer cells are members of the mononuclear phagocytic system and are derived from monocytes. In addition, pit cells are found, either in contact with endothelial cells or, more frequently, with Kupffer cells. Pit cells are considered as a subpopulation of peripheral blood mononuclear cells known to have natural killer activity, and adhering to the sinusoidal barrier.

Hepatic stellate cells, which account for about 5–8% of cells in the normal liver, are characterised by a perisinusoidal distribution in the Disse space and long processes extending around sinusoids and between the hepatocyte plates. Electron microscopy has shown that the nucleus-to-nucleus distance between two adjacent hepatic stellate cells is ~40 μm. Eight to ten hepatic stellate cells lie along each sinusoid, between the centrolobular vein and the portal tract. The close association of hepatic stellate cells with endothelial cells resembles that of pericytes in capillaries. In normal liver, very rare hepatic stellate cells express α-smooth muscle actin (i.e. are activated); interestingly, α-smooth muscle actin-expressing hepatic stellate cells are encountered surrounding dilated sinusoids (Fig. 2). Moreover, *ex vivo* liver perfusion induces (1) the early activation of hepatic stellate cells, which begin to produce α-smooth muscle actin, and (2) significant changes in the perisinusoidal extracellular matrix (Fig. 3) [26]. These findings are consistent with the view that hepatic stellate cells function as liver-specific pericytes, participating in the regulation of sinusoidal blood pressure. In normal liver, the endothelium is discontinuous and presents multiple fenestrations without diaphragms, allowing the rapid transport of solutes across the subendothelial space. In the normal liver, a basal lamina-like layer separates the two cell types but there is no true basement membrane. The sinusoidal endothelial cells do not express CD34 in normal conditions. Cytoplasmic lipid droplets containing vitamin A are present in about 75% of human hepatic stellate cells in normal liver [27], and hepatic stellate cells represent the largest cellular reservoir of vitamin A in the body. Hepatic stellate cells change phenotype under conditions of injury, often losing their lipid droplets upon activation. The biological markers of these cells also change according to activation level or position within the lobule. On activation, the hepatic stellate cells acquire a myofibroblastic phenotype, contributing to the extracellular matrix deposition observed in the pathological conditions of fibrosis and cirrhosis. During myofibroblastic differentiation, the hepatic stellate cells acquire the expression of α-smooth muscle actin (see above). In injured areas, soluble factors (cytokines) are released by incoming inflammatory cells, damaged and regenerating hepatocytes, and other liver cells inducing the activation of hepatic stellate cells. In fibrosis and cirrhosis, capillarisation of the sinusoids occurs, with formation of a continuous endothelial layer acquiring a true basal lamina.

Alcoholic hepatitis and non-alcoholic steatohepatitis (linked to insulin resistance and underlying metabolic syndrome – obesity, diabetes mellitus, hypertriglyceridaemia, etc.) are associated with the development of a particular type of fibrosis. Early fibrotic changes are concentrated in the centrolobular vein area around the sinusoids (i.e. perisinusoidal fibrosis) with capillarisation of the sinusoids.

Capillarisation of the sinusoids also occurs in cirrhosis, which is the endpoint of liver fibrosis, whatever the aetiology (Fig. 4). Cirrhosis is characterised by the formation of regenerative nodules of liver parenchyma separated by fibrotic septa. In sinusoidal structures, α-smooth muscle actin-positive myofibroblasts and CD34-expressing endothelial cells are well represented (Fig. 4). Three major mechanisms

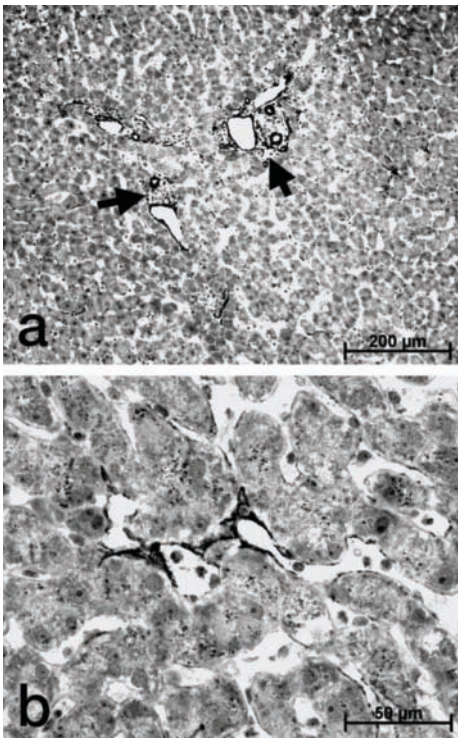

Figure 2
α-Smooth muscle actin expression in normal human liver.
In normal human liver, α-smooth muscle actin is expressed by smooth muscle cells in centrolobular veins and in portal vessels (veins and arteries, arrows) (a); in the parenchyma, rare hepatic stellate cells surrounding dilated sinusoids express α-smooth muscle actin (b).

are involved in the generation of cirrhosis: cell death, aberrant extracellular matrix deposition (fibrosis), and vascular reorganisation. Fibrous septa connecting the portal tracts and hepatic veins form, leading to portovenous and arteriovenous shunting, and bypassing of the parenchymal nodules. This results in vascular thrombosis of the medium-sized and large portal veins and of the hepatic veins and in the progression of parenchymal extinction, i.e. loss of continuous hepatocyte layers [28]. In most cases, significant lesions are observed only after months or years of injury. However, they may appear more rapidly in congenital liver diseases, such as biliary atresia. The poor prognosis of cirrhosis is aggravated by the frequent development of hepatocellular carcinoma, which may also occur, albeit much more rarely, in normal or only slightly fibrotic livers. In hepatocellular carcinoma, groups of tumoural hepatocytes are surrounded by α-smooth muscle actin-positive cells, and CD34-expressing endothelial cells (Fig. 5). In both advanced cirrhosis and hepato-

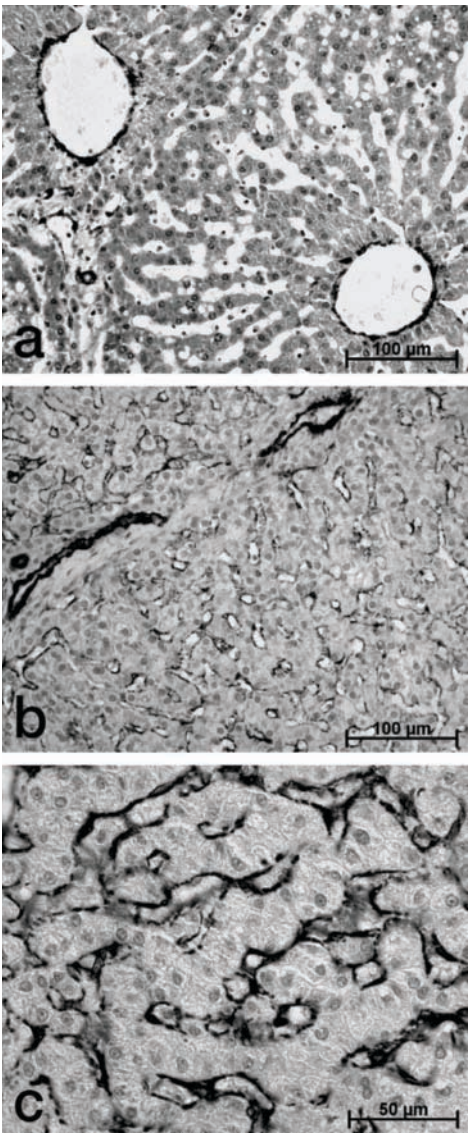

Figure 3
α-Smooth muscle actin expression in hepatic stellate cells during ex vivo *pig liver perfusion.*
In normal liver (a), α-smooth muscle actin is expressed by smooth muscle cells in centrolobular veins and portal vessels. After 1 h of perfusion (b), a strong reactivity for α-smooth muscle actin is present in most hepatic stellate cells, particularly along dilated sinusoids. A dotted line pattern caused by the α-smooth muscle actin containing processes of hepatic stellate cells is frequently observed (c).

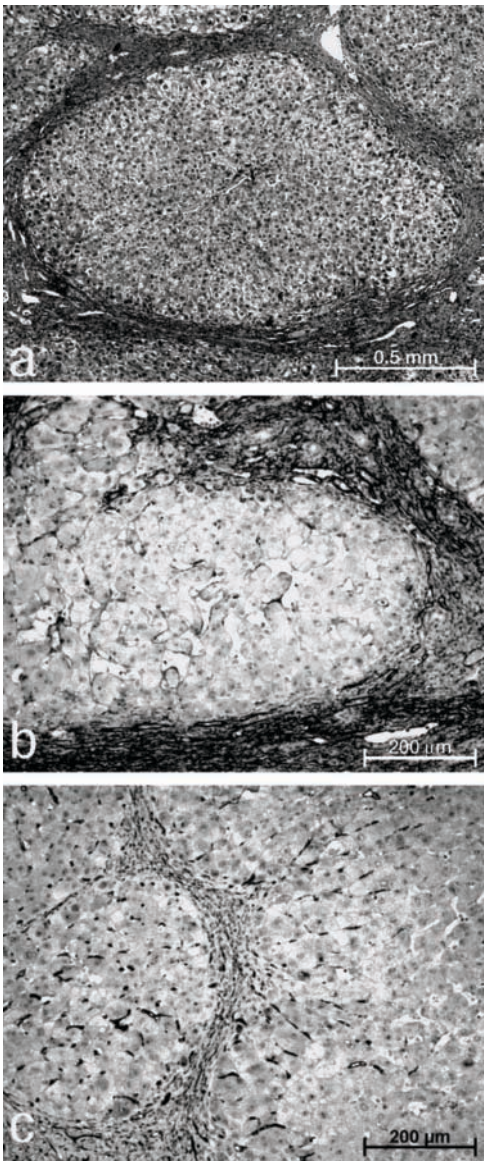

Figure 4
Modification of the liver organisation in human cirrhosis.
Haemalum-eosin-saffron histochemistry shows a typical nodule of regeneration surrounded by a fibrotic septa (a). α-Smooth muscle actin is expressed in vessel wall and in myofibroblasts present in the fibrotic septa, and in a few hepatic stellate cells in the parenchyma (b). Some endothelial cells express CD34, underlining the phenotypic modification of sinusoidal endothelial cells (c).

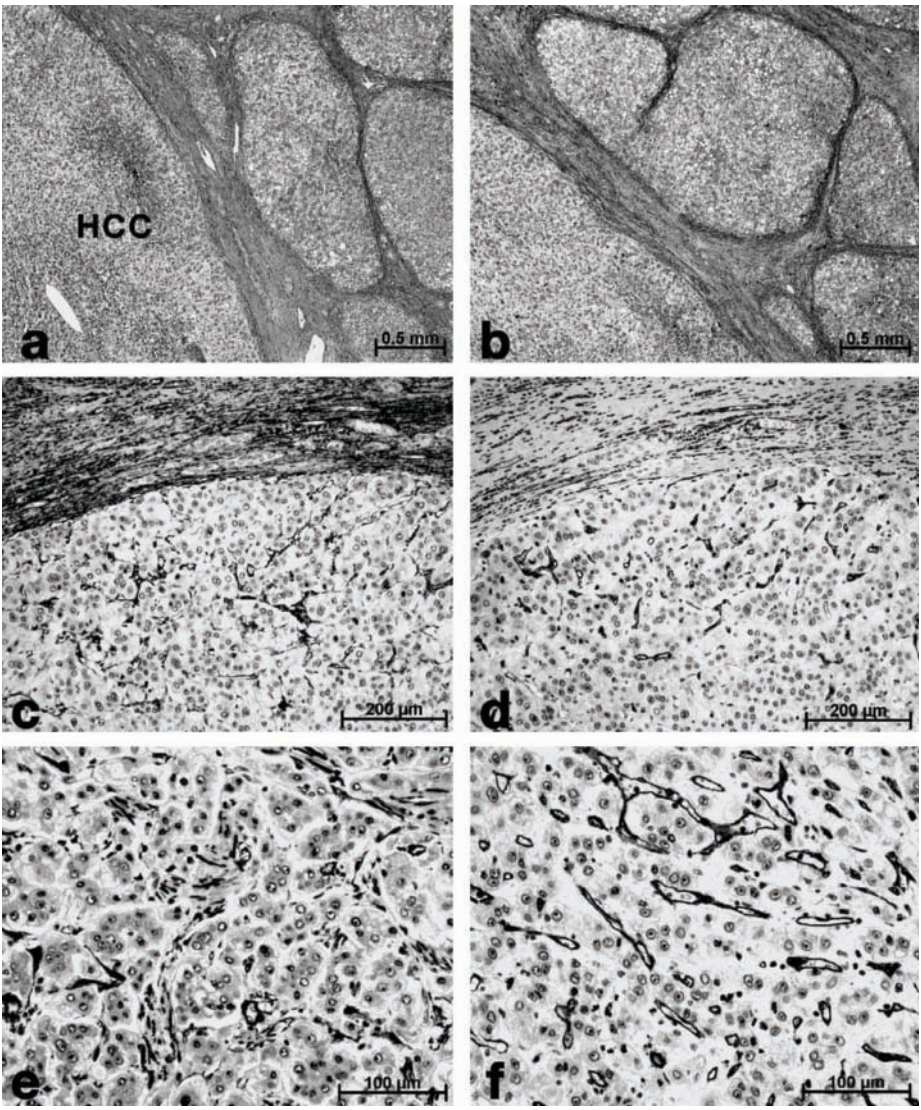

Figure 5
Hepatocellular carcinoma arising in a cirrhotic liver.
Haemalum-eosin-saffron (a) and Masson's trichrome (which stains fibrillar collagens) (b) histochemistry shows the stroma reaction surrounding the hepatocellular carcinoma (HCC) and the cirrhotic nodules surrounding the tumour lesion. The tumoural stroma resembles the fibrous stroma of the surrounding cirrhosis. At the periphery of the tumour lesion, in the stroma reaction, α-smooth muscle actin is present in vessel wall and in myofibroblasts (c), and CD34 is expressed in vascular endothelial cells (d). In the tumour, α-smooth muscle actin-expressing cells (c, e) and CD34-expressing endothelial cells (d, f) are observed.

cellular carcinoma, the origin of the cells expressing α-smooth muscle actin is not well known: it could be hepatic stellate cells expressing a myofibroblast phenotype, or smooth muscle cells or pericytes migrating from invading vessels coming from the perinodular fibrotic septa or from the stroma reaction [29]. Similarly, in both pathological situations, the origin of CD34-positive endothelial cells is also questionable: either sinusoidal endothelial cells lose their typical features (i.e. discontinuous layer and presence of fenestrations) and acquire a phenotype of vascular endothelial cells with the expression of CD34, or angiogenesis occurs with involvement of vascular endothelial cells growing from preexisting vessels present around the lesion.

Roles of the renin-angiotensin system

Following the seminal experiments of Goldblatt [30], in which the restriction of blood flow through the renal arteries was shown to produce a sustained increase of blood pressure, it has been established that this ischaemia-linked hypertension is produced by an enzymatic chain of events involving renin, in which a circulating pressor substance, angiotensin, is generated [31, 32]. Angiotensin II emerged as the most important effector peptide of the renin-angiotensin system (RAS), and its central physiological role in the regulation of salt homeostasis, kidney function, and blood pressure has been well established.

Angiotensin II, unlike other classical circulating peptide hormones, is not restricted to the blood and can be locally generated in almost all of the tissues of the body. For example, all components of the RAS are expressed in the heart where synthesis occurs in both parenchymal and connective tissue cells. Cultured myocytes and fibroblasts can also produce angiotensinogen [33]. It was shown that other peptides such as angiotensin III and angiotensin IV, initially regarded as inactive breakdown products of angiotensin II, are active on their own [34]. Finally, RAS acts through several receptors (Fig. 6) [35]. Particularly, two different receptors for angiotensin II, angiotensin type 1 (AT1) and angiotensin type 2 (AT2) receptors, have been characterised.

The complexity of RAS suggested a broader physiological role than merely salt homeostasis and blood pressure control. Indeed, it was demonstrated that it participates in many additional regulatory processes, such as inflammation, athero-thrombosis, cardiac and biological activities. Angiotensin-converting enzyme (ACE) and angiotensin receptors are expressed by mesenchymal cells responsible for connective tissue turnover, and it appears that RAS participates in connective tissue homeostasis [36]. It has been shown that myofibroblasts generate angiotensin II *de novo*. In fibrotic tissues, ACE, angiotensin II and TGF-β receptor are highly expressed where α-smooth muscle actin-expressing myofibroblasts secreting fibrillar collagens and TGF-β1 are present (Fig. 6) [36]. Experimental evidence also indicates that angiotensin II tends to restore blood flow in the setting of acute ischaemia and plays a role

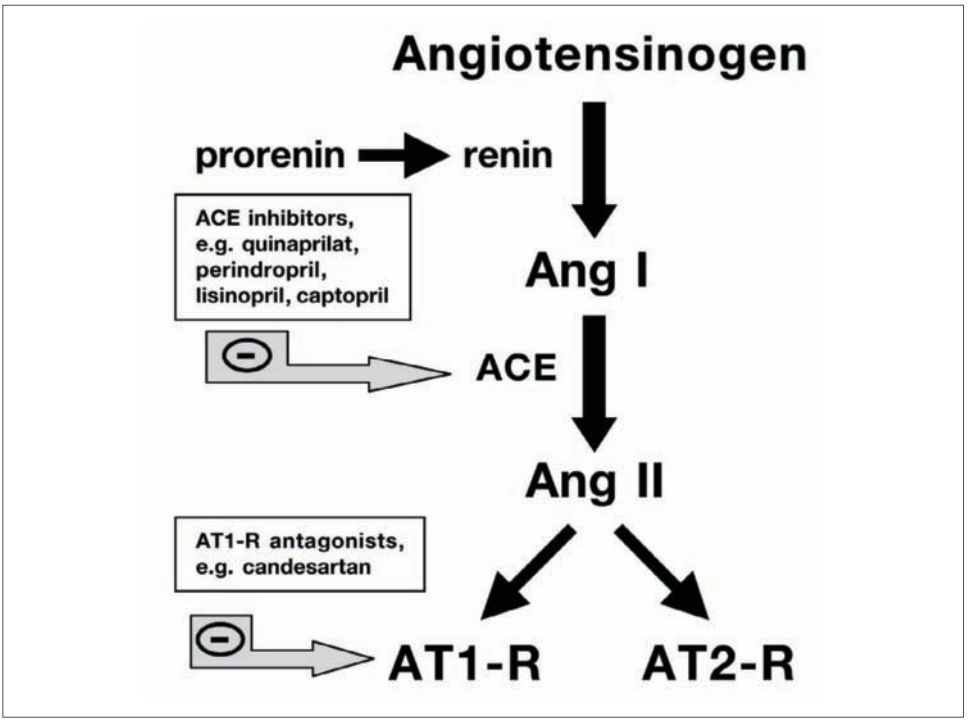

Figure 6
Renin-angiotensin system.
Myofibroblasts are able to produce angiotensinogen, angiotensin-concerting enzyme (ACE), and cathepsin D, which are involved in the generation of angiotensin (Ang) II; they also express angiotensin type 1 receptor (AT1-R). Cell-cell signalling between myofibroblasts involves Ang II and autocrine induction of TGF-β1; this is also the case between myofibroblasts and neighbouring endothelial cells where paracrine properties of Ang II contribute to the production of various signals including stimulators and inhibitors of extracellular matrix turnover (see [36]). ACE inhibitors and/or AT1-R antagonists could be used in the context of pathological tissue repair situations involving myofibroblasts.

in angiogenesis (a process crucial in maintaining tissue perfusion during sustained ischaemia [37]), in tissue repair and in tumoural neoangiogenesis.

Angiotensin II as angiogenic factor

Ilich et al. [38] reported that renal ischaemia induces the development of collateral circulation, but also an increase of endothelial cell turnover in perirenal vessels; a humoral angiogenic factor produced by the ischaemic kidney was implicated in

this effect. Cuttino et al. [39] found that, besides the development of the collateral circulation, a factor extracted from ischaemic kidneys induced the formation of new vessels when tested in the cheek pouch of the hamster. The effect of angiotensin II on vascular neoformation *in vivo* was subsequently described in the rabbit cornea [40]. The angiogenic effect of angiotensin II was further verified in other models of angiogenesis, such as the chorioallantoic membrane of the chick embryo and sponge implantation in the rodent [41–43]. In the rat cornea, the mechanism by which angiotensin II stimulates vessel growth appears to be related to a chemotactic effect of this compound on endothelial cells [44] and/or through the migration of pericytes [45]. The angiogenic effect of angiotensin II could be a direct action on endothelial cells or an indirect action through the expression of VEGF or FGF-2 [46, 47]. As underlined above, VEGF is one of the major angiogenic factors and many reports have indicated that VEGF activates new vessel formation in many tissue types. It has been reported that angiotensin II stimulates the expression of VEGF in vascular smooth muscle cells under non-hypoxic conditions [48]. This is caused by induction of HIF-1 by angiotensin II through the AT1 receptor. Angiotensin II increases HIF-1 gene expression through transcriptional and post-transcriptional mechanisms [49]. Angiotensin II-induced VEGF expression in endothelial cells is also mediated *via* AT1 receptor [50]. Experiments at the cellular level strongly support the idea that activation of AT1 receptor mediates angiogenesis. In mice lacking AT1 receptor, angiogenesis induced by hind-limb ischaemia was impaired compared with wild-type mice [51]. Although AT1 receptor-deficient mice show substantial decrease in blood pressure compared with wild-type mice, the effect was blood pressure independent because reduction of blood pressure comparable to AT1 receptor-deficient mice did not affect angiogenesis in wild-type mice. Candesartan, one of the clinically used AT1 receptor antagonists, at doses that did not affect blood pressure level, also inhibited angiogenesis in wild-type mice after hind-limb ischaemia. These two different approaches to inhibit AT1 receptor signal confirm that activation of AT1 receptor is pro-angiogenic.

A number of studies suggest, in contrast, that angiotensin II has anti-angiogenic properties. Inhibition of ACE by quinaprilat induced angiogenesis similarly to VEGF in a hind-limb ischaemic model [52]. Silvestre et al. [53] reported that the pro-angiogenic effect of ACE inhibitors was mediated through the bradykinin B2 receptor pathway, because perindopril (ACE inhibitor) enhanced reparative angiogenesis induced by hind-limb ischaemia in wild-type mice but not in bradykinin B2 receptor-knockout mice. These apparent discrepancies may simply reflect the actual complexity of RAS regulation. Recent research has shown that cell growth and proliferation are mediated by AT1 receptors, whereas stimulation of AT2 receptors leads to an inhibition of cell proliferation and promotes cell differentiation [54–56]. Under physiological conditions, AT1 and AT2 receptors develop sequentially during microvascular maturation, and the role of the endogenous angiotensin system in angiogenesis depends on the balanced local expression of its various components [57].

Angiotensin II and tumoural angiogenesis

Several reports have described the production of renin by both benign and malignant non-renal tumours [58–62]. There appears to be a correlation between the presence of renin and the degree of vascularisation in some tumours such as angiolymphoid hyperplasia with eosinophilia, glioblastoma, alveolar sarcoma, small cell carcinoma, and adenocarcinoma of lung, pancreas, and ovary, suggesting that renin, most likely through the cascade to angiotensin II, could contribute to angiogenesis. In rats implanted with Walker 256 carcinosarcoma, a dose-dependent reduction in tumour size was seen with lisinopril (ACE inhibitor). Moreover, another ACE inhibitor, captopril, curtails the growth of chemically induced and implanted tumours in rats and mice by reducing angiogenesis [44]. In another report, perindopril inhibited angiogenesis and metastasis of hepatocellular carcinoma with concomitant reduction of VEGF expression [63]. The tumoural angiogenic effect of angiotensin II could be mediated by the AT1 receptor, which is expressed on tumour-associated macrophages and mediates VEGF expression and angiogenesis. Indeed, tumour-associated angiogenesis is impaired in the AT1 receptor-deficient mice [64].

In a retrospective large cohort study based on the records of 5207 Scottish patients, Lever et al. [65] observed that the relative risks of incident and fatal cancer in the 1559 patients receiving ACE inhibitors were, respectively, 0.72 and 0.65 when compared with a control population in West Scotland, thereby providing the first clinical evidence supporting the concept that long-term use of ACE inhibitors may protect against cancer.

Conclusion

The fibroblast/myofibroblast transition is accepted as the key event in the formation of granulation tissue during wound healing or fibrotic changes and during the evolution of the stroma reaction (for review, see [9]). In these situations, important interactions between angiogenesis and fibroblast/myofibroblast evolution develop; inflammatory cells are also involved in the dialogue between vascular cells and fibroblasts/myofibroblasts. In addition to the soluble factors involved in these interactions, extracellular matrix, which can also transmit signals to different cell types, plays a major role. Together with blood vessels and infiltrating inflammatory cells, (myo)fibroblasts and the extracellular matrix represent the “benign” tumour compartment. Myofibroblasts secrete numerous angiogenic factors, including VEGF-A, the main regulator of angiogenesis under normal and pathological conditions. The role of myofibroblasts in angiogenesis is particularly crucial in the tumour stroma; reduction of angiogenesis in tumours is a therapeutic option and numerous works have evaluated the effects of diverse products acting on angiogenesis that are also able to decrease tumour growth. It has been recently shown that a cross-talk

between malignant epithelial cells and adjacent stromal cells leads to the development of a tumour microenvironment that promotes tumour progression. In a mouse model of prostate carcinoma, tumour cells induce up-regulation of p53 through a paracrine mechanism in stromal (myo)fibroblasts, which results in decreased (myo)fibroblast proliferation. This process creates a selective pressure that promotes the expansion of a highly proliferative subpopulation of (myo)fibroblasts that lack p53 and which contribute to tumour progression [66]. Coevolution of the stromal compartment with selection of genetically altered cells can then occur as a result of oncogenic stress in the epithelium. It would be interesting to study if these myofibroblastic subpopulations present specific effects on angiogenesis. In addition, depending on the tissue, different subpopulations of fibroblastic cells can be involved in the formation of myofibroblastic cells, and the interactions between vascular cells and myofibroblasts can vary according to the tissue environment. Particularly in the liver, the respective roles of hepatic stellate cells and of portal fibroblasts in angiogenesis development during hepatic carcinoma development remain to be clarified. The specific anatomical localisation of hepatic stellate cells in the perisinusoidal space favours interactions with sinusoidal endothelial cells. However, during sinusoid capillarisation, the respective role of sinusoidal cells (hepatic stellate and endothelial cells) and of growing capillaries derived from the stroma reaction, is not well defined. Finally, in this review, the roles of the RAS in the interactions between fibroblasts/myofibroblasts and vascular cells have been underlined. Myofibroblasts are able to produce angiotensinogen, ACE, and cathepsin D, which are involved in the generation of angiotensin II; they also express AT1 receptor. Contradictory observations have been published on the role of angiotensin II in angiogenesis. Moreover, numerous drugs have been developed, acting at different levels in the RAS system, which are being now tested for their action during fibrogenesis and tumour development.

All these observations underline the potential importance of therapies that target the fibroblast/myofibroblast compartment as a means to prevent acceleration or possibly suppress tumorigenesis. It has been shown that the selective inhibition of α-smooth muscle actin incorporation into stress fibres, which is the hallmark of myofibroblastic differentiation, by the administration of its N-terminal sequence NH_2-EEED results in reduction of the tension exerted by cultured myofibroblasts on their substratum coupled with a significant decrease of collagen type I synthesis by the same cells [67]. Moreover, this sequence, administered as a fusion peptide (FP) with a cell-penetrating sequence, produces a significant reduction of the contractile capacity of granulation tissue strips after endothelin-1 stimulation and a significant delay of wound contraction in rat wounds splinted for 10 days and treated for the last 3 days with the FP [67]. The action of myofibroblasts in influencing angiogenesis could be exerted through mechanical remodelling of perivascular environment and transmission of tension to vascular cells with a mechanism involving tension production by α-smooth muscle actin containing stress fibres,

and transmission of this force to the tissue through adhesion complexes connecting myofibroblasts to the extracellular matrix and to other cells (for review, see [4]). The availability of different tools such as this FP furnishes exciting opportunities for testing the extent of myofibroblasts participation in the development of blood vessels.

The concept of myofibroblast has generated a significant amount of research during the last 30 years. It appears that the myofibroblast plays remodelling functions that are necessary during repair phenomena in association with important mechanisms such as angiogenesis. But many aspects of myofibroblast biology are not yet clear. During the next few years, new findings will certainly represent the basis for new strategies aiming at the development of therapeutic tools for several important diseases.

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Chemokines and cytokines in inflammatory angiogenesis

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Introduction

The perpetuation of angiogenesis is involved in certain chronic inflammatory conditions such as rheumatoid arthritis (RA). Thus, RA, a prototype of chronic inflammatory disorders, as well as other inflammatory diseases with accelerated neovascularisation may be considered as "angiogenic diseases". Angiogenesis plays an important role in the pathogenesis of the disease and therapeutic control of angiogenesis may be beneficial for the outcome of inflammation (reviewed in [1–6]).

In RA, inflammatory leucocytes migrate through the fenestrated endothelia of vessels into the rheumatoid synovium. Leucocyte-vessel interactions are mediated by numerous cell adhesion receptors including integrins and selectins. Leucocyte adhesion and their transendothelial migration also involve inflammatory chemokines that drive inflammatory leucocytes through the endothelial barrier [3, 7]. Pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, IL-15, IL-18 and others have been implicated in the regulation of chemokine production, adhesion molecule expression and thus leucocyte ingress into the synovium [3, 5, 8]. There is intensive neovascularisation in the inflamed synovium, indicated by the great number of newly formed vessels. Enhanced angiogenesis results in an expanded endothelial surface and thus the augmentation of inflammatory cell adhesion and migration. Synovial macrophages and angiogenic mediators produced by these cells are key players in this process [7, 9]. RA-associated angiogenesis include numerous pro-inflammatory cytokines, growth factors, chemokines, components of the extracellular matrix, matrix-degrading enzymes, cellular adhesion molecules and others [3–6]. Most of these mediators have been detected in the RA synovium, are released by synovial endothelial cells and macrophages, and have been implicated in the pathogenesis of RA itself, as well as in RA-associated neovascularisation [1–3, 5, 6, 10, 11]. On the other hand, some anti-inflammatory cytokines and chemokines, as well as other compounds inhibit synovial neovascularisation and thus may suppress arthritis [4, 5].

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In addition to pathogenetic aspects, angiogenesis research has important clinical relevance. For example, small molecular agents and biologicals that suppress synovial angiogenesis may, in theory, be included in the management of RA and other types of arthritis [1–6].

In this review we discuss recent information on the role of inflammatory chemokines and cytokines in the pathogenesis of inflammation-associated neovascularisation. Our demonstration is mostly based on results obtained in inflammatory arthritis, which has been widely characterised with respect to chemokines, cytokines and angiogenesis in recent years. In addition, we give examples of recent advances in specific angiogenesis inhibition in inflammatory diseases.

Inflammatory chemokines in angiogenesis

Chemokines and chemokine receptors

Chemokines are small proteins exerting chemotactic activity towards leucocytes and other cell types. Target cells express matching receptors for these mediators. Chemokines have been classified into supergene families with respect to their structure (reviews: [3, 12–15]) (Tab. 1). According to the location of cysteine (C) residues, these families are designated as CXC, CC, C and CX_3C chemokines (Tab. 1). Accordingly, the four chemokine receptor groups are CXCR, CCR, CR and CX_3CR, respectively [3, 12–15] (Tab. 1). Currently, there are more than 50 known chemokines and 19 chemokine receptors (reviews: [3, 12–15]). Some years ago, a new classification system was introduced. Chemokines are now considered as chemokine ligands, and, apart from their classical name, each chemokine has been assigned a designation of CXCL, CCL, XCL or CX_3CL1 [15] (Tab. 1). In this review, both the classical and the new designations will be used.

In addition to this structural classification, chemokine/chemokine receptor pairs have also been functionally categorised as being "homeostatic" (alternatively: constitutive, housekeeping or lymphoid) or "inflammatory" (alternatively: inducible). "Homeostatic" chemokines usually play a role in B cell recruitment, germinal centre formation and the development of lymphoid tissues under physiological conditions. All chemokines described in context with arthritis may be considered to be "inflammatory". It is also safe to say that numerous inflammatory chemokines also modulate neovascularisation. However, all these functions often overlap and, as described later, some "homeostatic" chemokines have also been implicated in the pathogenesis of inflammation including RA (reviews: [16, 17]). For the sake of brevity and clarity, structural and functional details are not presented on all inflammatory chemokines and their receptors (see Tab. 1 instead), only their possible involvement in angiogenesis is discussed.

Table 1 - Chemokine receptor-ligand pairs involved in inflammatory angiogenesis.

Chemokine receptor	Chemokine ligand
CXC chemokine receptors	
CXCR1	IL-8/CXCL8*, GCP-2/CXCL6
CXCR2	IL-8/CXCL8*, ENA-78/CXCL5*, Groα/CXCL1*, Groβ/CXCL2, Groγ/CXCL3, CTAP-III/CXCL7*, GCP-2/CXCL6
CXCR3	IP-10/CXCL10*, PF4/CXCL4*, Mig/CXCL9*, ITAC/CXCL11
CXCR4 (fusin)	SDF-1/CXCL12*
CXCR5	BCA-1/CXCL13
CXCR6	CXCL16
C-C chemokine receptors	
CCR1	MIP-1α/CCL3, RANTES/CCL5, MCP-3/CCL7, HCC-1/CCL14, HCC-2/CCL15, HCC-4/CCL16, LD78β/CCL3L1, MPIF-1/CCL23
CCR2	MCP-1/CCL2*, MCP-3/CCL7, MCP-4/CCL13, HCC-4/CCL16
CCR3	Eotaxin/CCL11, Eotaxin-2/CCL24, Eotaxin-3/CCL26, RANTES/CCL5, MCP-2/CCL8, MCP-3/CCL7, MCP-4/CCL13, HCC-2/CCL15, MEC/CCL28
CCR4	TARC/CCL17, MDC/CCL22, CKLF1
CCR5	MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, LD78β/CCL3L1, MCP-2/CCL8, HCC-1/CCL14
CCR6	MIP-3α/CCL20
CCR7	MIP-3β/CCL19, SLC/6Ckine/CCL21*
CCR8	I-309/CCL1
CCR9	TECK/CCL25
CCR10	CTACK/CCL27, MEC/CCL28
C chemokine receptors	
XCR1	Lymphotactin/XCL1, SCM-1β/XCL2
C-X3-C chemokine receptors	
CX3CR1	Fractalkine/CX_3CL1*
Other	
DARC*	Duffy antigen, some CC and CXC chemokines

**Involved in angiogenesis. See text for abbreviations*

Among CXC chemokines, IL-8/CXCL8, epithelial-neutrophil activating protein-78 (ENA-78)/CXCL5, growth-related gene product α (groα)/CXCL1, connective tissue activating protein III (CTAP-III)/CXCL7, granulocyte chemotactic protein 2 (GCP-2)/CXCL6, interferon (IFN)-γ-inducible protein 10 (IP-10)/CXCL10, monokine induced by IFN-γ (Mig)/CXCL9, platelet factor 4 (PF4)/CXCL4, stromal cell-derived factor-1 (SDF-1)/CXCL12 and B cell activating chemokine 1 (BCA-1)/CXCL13 and, recently, CXCL16 have been implicated in RA. Thus, these chemokines may be considered "inflammatory" [3, 12, 18, 19]. Some CXC chemokines promote, while others inhibit, angiogenesis [3, 14, 20]. In general, the angiogenic or angiostatic action of these mediators greatly depends on the Glu-Leu-Arg (ELR) sequence. ELR-containing chemokines, such as IL-8/CXCL8, ENA-78/CXCL5, groα/CXCL1, and CTAP-III/CXCL7 stimulate vessel formation. In contrast, CXC chemokines lacking the ELR sequence, such as PF4/CXCL4, IP-10/CXCL10 and Mig/CXCL9 are angiostatic [3, 14, 20]. However, as an exception to the rule, the ELR-lacking SDF-1/CXCL12 is angiogenic [3, 20].

Some CC chemokines, such as MCP-1/CCL2 and others have been implicated in the pathogenesis of RA and other inflammatory disorders. However, as described below, very few data are available regarding the role of CC chemokines in angiogenesis [21, 22].

The C chemokine family contains two members: lymphotactin/XCL1 and single C motif 1β (SCM-1β)/XCL2. The CX_3C subset contains a single member: fractalkine/CX_3CL1 [3, 23–25].

Chemokines described above mediate their effects *via* seven-transmembrane domain receptors expressed on the target cells [15]. There is significant redundancy between CXC and CC chemokine receptors and their ligands (Tab. 1). For example, CXCR2, CCR1 or CCR3 have numerous chemokine ligands, while CXCR6, CCR8 or CCR9 are specific receptors for one single ligand [3, 15] (Tab. 1). There is only one C and CX_3C chemokine receptor for their respective chemokine ligands [23, 24] (Tab. 1). CXCR2, a receptor for most ELR motif-containing CXC chemokines, plays a crucial role in inflammation and angiogenesis. In contrast, CXCR3 is a receptor for most ELR-lacking, angiostatic CXC chemokines [3, 15, 20] (Tab. 1).

Chemokines in angiogenesis

IL-8/CXCL8 is one regulator of angiogenesis in RA. This ELR-containing chemokine is chemotactic and mitogenic for vascular endothelial cells *in vitro* [3, 12, 14, 20]. Endothelia express CXCR2, a receptor for IL-8/CXCL8 [3, 12, 21]. ENA-78/CXCL5 exerts angiogenic activity in RA [1, 14]. CTAP-III/CXCL7 also induces angiogenesis [3, 26]. Groα/CXCL1 has recently been implicated in thrombin-induced angiogenesis [27]. In addition, the inflammatory mediator prostaglandin E_2 induces groα/CXCL1 expression and neovascularisation mediated by this che-

mokine [28]. All these CXC chemokines have been detected in the sera and synovia of RA patients [3, 12].

IP-10/CXCL10 exerts proinflammatory, but anti-angiogenic effects in RA [3, 12, 20, 29]. This ELR-lacking chemokine has been shown to suppress neovascularisation [14, 20]. In contrast, a recent study suggests that the effect of VEGF on endothelial cells is, at least in part, mediated by IP-10/CXCL10 as VEGF induces endothelial expression of IP-10/CXCL10 protein and mRNA [29]. On the other hand, IP-10/CXCL10 inhibits VEGF-induced endothelial motility on Matrigel. This suppressing action involves calpain inhibition [30]. Thus, VEGF and IP-10/CXCL10 may form an autocrine loop: VEGF induces the production of IP-10/CXCL10, and the chemokine, in turn, suppresses VEGF-induced angiogenesis [29, 30]. Mig/CXCL9 and PF4/CXCL4 also lack the ELR motif and thus these chemokines are angiostatic [3, 20]. All these chemokines have been detected in RA [12].

SDF-1/CXCL12 is a specific ligand for CXCR4. SDF-1/CXCL12, despite lacking the ELR motif, promotes neovascularisation [31, 32]. This chemokine induced endothelial chemotaxis *in vitro* and dermal angiogenesis in mice *in vivo* [32]. RA synovial fibroblasts abundantly produce SDF-1/CXCL12 under hypoxic conditions. In this situation, SDF-1/CXCL12 becomes immobilised on endothelial heparan sulphate, where this chemokine is able to promote angiogenesis [31]. Circulating human $CD34^+$ cells expressing the VEGF-2 receptor have been identified and characterised [33]. This subpopulation of cells consists of functional endothelial precursors playing a role in angiogenesis [33]. In addition, virtually all $CD34^+$/VEGF-2 $receptor^+$ cells express CXCR4 and migrate in response to SDF-1 [33]. As recently shown, cytokine-mediated release of SDF-1/CXCL12 induces revascularisation through the recruitment of $CXCR4^+$ "haemangiocytes" [34]. Thus, SDF-1 may be the first angiogenic CXC chemokine that lacks the ELR motif. Although it is generally believed that SDF-1/CXCL12 has a single receptor, CXCR4, very recently, another alternative receptor for this chemokine, as well as for IFN-inducible T cell α chemoattractant (I-TAC)/CXCL11, has been implicated in SDF-1/CXCL12-mediated angiogenesis and tumour growth [35]. SDF-1/CXCL12 expression may be a prognostic marker in low-grade gliomas [36].

Fewer data are available on the possible role of CC chemokines in angiogenesis. Some years ago, Salcedo et al. [21] reported that MCP-1/CCL2 induced endothelial chemotaxis *in vitro*, as well as angiogenesis in the chick chorioallantoic membrane assay *in vivo*. MCP-1/CCL2-induced neovascularisation was associated with abundant endothelial expression of CCR2 [21]. Recently, the direct angiogenic activity of MCP-1/CCL2 has been confirmed [22]. This chemokine acts *via* the up-regulation of the Ets-1 transcription factor, and activation of Ets-1 involves integrins and the activation of the ERK-1/2 cascade [22]. Recent reports also suggest that MCP-1/CCL2 is an inflammatory "arteriogenic" factor. Using murine models of fibroblast growth factor (FGF)-2-mediated therapeutic neovascularisation, FGF-2 was shown to enhance both VEGF-mediated angiogenic and MCP-1/CCL2-driven arteriogenic

signals *via* independent signalling pathways [37]. Thus, MCP-1/CCL2 may act in concert with other angiogenic mediators during vasculogenesis.

Vicari et al. [38] studied secondary lymphoid tissue chemokine (SLC/CCL21). This chemokine showed strong angiostatic and anti-tumour effects. Recently, MPIF-1/CCL23 has been implicated in the migration of vascular endothelial cells and matrix metalloproteinase production [39]. Yet, the role of MCP-1/CCL2 and other CC chemokines in angiogenesis needs further confirmation.

Fractalkine/CX_3CL1 has been implicated in neovascularisation and atherosclerosis [25]. CX_3CR1-deficient mice showed attenuated development of atherosclerosis [40]. In humans, an M280/I249 polymorphism in the CX_3CR1 gene was associated with reduced cardiovascular risk [41]. Fractalkine/CX_3CL1 is also angiogenic [25]. As accelerated atherosclerosis and increased cardiovascular risk is the primary cause of death in RA patients, these results may have important clinical relevance.

Chemokine receptors in neovascularisation

Among CXC chemokine receptors, CXCR2 recognises the most important proinflammatory and pro-angiogenic CXC chemokines described above [3, 12] (Tab. 1). CXCR2 is expressed on RA macrophages, neutrophils, as well as articular chondrocytes [3, 42]. CXCR2 is also expressed by the inflammatory endothelium and thus play a role in chemokine-induced angiogenesis [3, 12, 21]. As discussed above, CXCR4, is the specific receptor for SDF-1/CXCL12, and thus it may be implicated in SDF-1/CXCL12-induced neovascularisation in RA [18, 19]. The possible role of CXCR7 recognising I-TAC/CXCR11 and SDF-1/CXCR12 in angiogenesis is described above [35].

As shown in Table 1, Duffy antigen/receptor for chemokines (DARC) cannot be classified into one of the four classical chemokine receptor subclasses. DARC, originally described on erythrocytes, binds the Duffy antigen, as well as some CXC and CC chemokines. Recently, DARC expression has been detected on RA synovial endothelium [43]. DARC has also been implicated in breast cancer-associated angiogenesis [44].

As the angiostatic IP-10/CXCL10 and MIG/CXCL9 bind to CXCR3, this receptor may be involved in chemokine-mediated angiostasis [3].

Angiogenic and angiostatic pro-inflammatory cytokines and growth factors

Some relevant mediators implicated in RA-associated angiogenesis are included in Table 2. Some of the more important molecules are discussed in more details.

Table 2 - Some angiogenic mediator and inhibitor chemokines, growth factors and cytokines in inflammation.

	Mediators	**Inhibitors**
1. Growth factors	bFGF, aFGF	TGF-β*
	VEGF	
	HGF	
	PDGF, PD-ECGF	
	EGF	
	IGF-1	
	HIF-1, HIF-2	
	TGF-β*	
2. Cytokines	TNF-α	IL-1*
	IL-1*	IL-4
	IL-6*	IL-6*
	IL-15	IFN-α, IFN-γ
	IL-17?	IL-12
	IL-18	LIF
	G-CSF, GM-CSF	
	Oncostatin M	
3. Chemokines	IL-8/CXCL8	PF4/CXCL4
	ENA-78/CXCL5	IP-10/CXCL10
	Groα/CXCL1	MIG/CXCL9
	CTAP-III/CXCL7	SLC/CCL21
	SDF-1/CXCL12	
	MCP-1/CCL2	
	MPIF-1/CCL23	

**May exert both angiogenic and angiostatic properties.*

Growth factors

VEGF is probably the most well-known angiogenic factor associated with RA and other types of chronic inflammation [45]. VEGF protein and mRNA are expressed in the arthritic synovium [3, 45]. VEGF has long been implicated in endothelial cell migration, proliferation, and chemotaxis, and thus neovascularisation [1, 2, 5, 46]. As described above, there may be two-way relationship between the action of VEGF

and IP-10/CXCL10 [29, 30]. As the RA synovium contains fenestrated endothelia, it may be relevant that VEGF also induces endothelial fenestration [47]. The role of $CD34^+$ blood stem cells carrying VEGF receptors termed "haemangiocytes" in angiogenesis and vasculogenesis is discussed later [3, 33].

Basic FGF (bFGF), acidic FGF (aFGF) and hepatocyte growth factor (HGF)/scatter factor are bound to heparin and heparan sulphate in the synovial interstitium. These growth factors are mobilised by heparanase and plasmin during neovascularisation [1, 5]. All these growth factors were detected in inflamed synovia [8, 48, 49].

Hypoxia is an important feature within the RA joint. Hypoxia itself stimulates the production of VEGF. In addition, hypoxia-inducible factors (HIF-1α and HIF-2α) that regulate VEGF gene transcription have also been implicated in angiogenesis [2, 3, 5].

Some growth factors that do not bind to heparin may also stimulate neovascularisation under inflammatory conditions. These include platelet-derived growth factor (PDGF), platelet-derived endothelial cell growth factor (PD-ECGF)/gliostatin, epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I) and transforming growth factor-β (TGF-β) [1, 5]. Among these growth factors, TGF-β may exert dose-dependent stimulatory and inhibitory effects on angiogenesis [3, 5]. These growth factors are also produced in chronic inflammatory states, such as RA [3, 8, 50].

Cytokines

Among pro-inflammatory cytokines, TNF-α, IL-1, IL-6, IL-15, IL-18 and possibly IL-17 are involved in angiogenesis, as well as in the pathogenesis of RA [2, 3–5, 51–54]. Other angiogenic cytokines include granulocyte (G-CSF) and granulocyte-monocyte (GM-CSF) colony-stimulating factors, as well as oncostatin M [2–5]. These cytokines are abundantly produced in the sera and synovia of RA patients [1, 5]. Among pro-inflammatory cytokines, TNF-α may account for a significant portion of macrophage-derived angiogenic activity in RA [55]. IL-1 has been described as a potent angiogenic mediator in the Matrigel assay [52]. IL-1-dependent angiogenesis requires the presence of macrophages and cyclooxygenase-2 (COX-2), as the removal of macrophages or a COX-2 inhibitor reduces neovascularisation in this system [56]. The role of IL-6 in angiogenesis is somewhat controversial as this cytokine may, under circumstances, stimulate or inhibit neovascularisation [2, 3, 5]. Recently identified cytokines, such as IL-15 and IL-18 also induce blood vessel growth [51–53]. In a human non-small cell lung cancer model IL-17 promoted CXCR2-dependent angiogenesis [54]. G-CSF and GM-CSF exert mild, less pronounced angiogenic activities in comparison to growth factors described above [2, 3, 5]. Oncostatin M stimulates neovascularisation *via* bFGF [57]. All these cytokines have been detected in the RA synovium [2, 3, 5, 8, 9]

Other cytokines, such as IFN-α, IFN-γ, IL-4, IL-12 and leukaemia-inhibitory factor (LIF) indirectly suppress neovascularisation by blocking the secretion of angiogenic cytokines and chemokines [1, 4, 5, 58, 59]. IFNs block bFGF- and VEGF-independent angiogenesis [1, 4, 5]. IL-4 antagonises the angiogenic effects of TNF-α and IL-1 [58]. IL-12 blocks angiogenesis by inducing the production of the angiostatic IFN-γ and IP-10/CXCL10 [59]. LIF is also angiostatic [60]. As described above, IL-6 may either stimulate or suppress angiogenesis [2, 3, 5]. All of these cytokines are detectable in the inflamed synovium [2, 3, 5, 60].

Regulation of inflammatory angiogenesis in the synovium

The outcome of inflammatory angiogenesis and thus leucocyte recruitment into the synovium depends on the imbalance between angiogenic mediators and angiostatic factors. A regulatory network consisting of numerous interactive mechanisms involving inflammatory chemokines, cytokines and other mediators exists in the RA synovium [1–6]. For example, pro-inflammatory cytokines, such as TNF-α, stimulate chemokine production thus perpetuating angiogenesis: TNF-α and IL-1 stimulate RA synovial fibroblasts to produce angiogenic chemokines and growth factors. Pro-inflammatory cytokines can also up-regulate the expression of endothelial, angiogenic adhesion molecules [1, 3, 5]. Interactions between VEGF and HIFs or IP-10/CXCL10 have been described above [2, 3, 29, 30]. Soluble and cell surface-bound angiogenic factors may also interact with each other: VEGF, in part, acts *via* integrin-dependent pathways [2, 5]. Other regulatory mechanisms include the balance between specific antagonistic pairs, such as ELR-containing *versus* ELR-lacking chemokines, pro-inflammatory, angiogenic (e.g. TNF-α, IL-1) *versus* anti-inflammatory, angiostatic (e.g. IL-4, interferons) cytokines [3, 5, 12, 14, 20]. As described above, TGF-β and IL-6, may stimulate or inhibit angiogenesis depending on their concentration. Thus, the production of these mediators may be a key regulator of neovascularisation [1, 3, 5]. The use of synthetic compounds including disease-modifying anti-rheumatic drugs (DMARDs) to inhibit neovascularisation is also a method of regulation, which is also relevant for anti-angiogenic therapy [1, 3, 5].

The possible prognostic value of angiogenesis in inflammatory diseases

Angiogenesis research may have important practical prognostic relevance. For example, the number of newly formed blood vessels in biopsy specimens may reflect the progression of the disease, similar to that described in malignancies [1, 2, 5]. Significantly higher degrees of vascularity have been detected in RA in comparison to osteoarthritic or normal synovial tissues [3, 8]. The synovial expression of some

angiogenic mediators may also exert prognostic value. For example, as described above, the expression of SDF-1/CXCL12 in gliomas correlated with tumour progression [36]. Thus, the synovial expression of certain inflammatory chemokines and cytokines may also be correlated with synovial angiogenesis and the progression of inflammation.

Inhibition of angiogenic and use of angiostatic chemokines and cytokines to control chronic inflammation

Angiogenesis inhibitors

As described above, some cytokines and chemokines including IL-4, IL-12, IFN-α, IFN-γ, as well as ELR-lacking chemokines suppress neovascularisation [1, 3, 5, 10–12]. Many of these factors also influence the progression of RA and thus, they may be useful for the management of this disease.

A number of anti-rheumatic agents currently used for the treatment of RA have been shown to suppress endothelial cell migration and angiogenesis. These compounds, including dexamethasone, gold salts, chloroquine, sulphasalazine, methotrexate, azathioprine, cyclophosphamide, leflunomide, thalidomide, minocycline, anti-TNF agents and possibly cyclosporine A, act, at least in part, by the inhibition of angiogenic chemokine and cytokine production [1, 4, 5, 8, 12]. For example, corticosteroids suppress the production of IL-8/CXCL8 by synovial cells [61, 62]. Sulphapyridine, a constituent of sulphasalazine, inhibited cytokine-stimulated endothelial cell expression of IL-8/CXCL8 [63]. Anti-TNF-α monoclonal antibody blockade reduced synovial expression of IL-8 and angiogenesis in RA patients [64].

Some antibiotics and their derivatives may suppress angiogenesis *via* the inhibition of VEGF. Apart from minocycline mentioned above, TNP-470, an angiostatic analogue of fumagillin, a naturally occurring product of *Aspergillus fumigatus*, decreases serum levels of VEGF and inhibits angiogenesis [1, 4, 5].

Angiogenesis targeting

As discussed above, currently used anti-rheumatic agents including classical DMARDs and anti-TNF biologicals, among other anti-inflammatory effects, also suppress synovial angiogenesis. For example, infliximab treatment reduced synovial VEGF expression and vascularity [5, 64]. Other angiogenic cytokines, such as IL-1 and IL-6 have also been targeted in biological therapy [8, 65, 66].

Future anti-angiogenic therapy, which also controls synovial inflammation in RA, may also target growth factors, chemokines and cytokines described above.

There have been attempts to target VEGF. A number of synthetic VEGF and VEGF receptor inhibitors, and anti-VEGF antibodies are under development [66, 67]. A soluble VEGF receptor 1 chimeric protein dose-dependently inhibited the proliferation of endothelial cells isolated from arthritic synovial tissues [67]. A humanised antibody to VEGF suppressed neovascularisation [67]. Among angiostatic chemokines, PF4/CXCL4 has been tried in animal models of arthritis [4, 5]. Combination of MIG/CXCL9 chemokine gene therapy with cytotoxic agents improved the therapeutic efficacy of the latter drug in cancer [68]. Blockade of the angiogenic chemokine receptor CXCR2 inhibited tumour-induced angiogenesis [69]. In general, most angiostatic agents may have therapeutic relevance for arthritis-associated angiogenesis and many are already in pre-clinical therapeutic trials.

It is likely, that multipotent rather than specific immunotherapy may be useful for the therapy of RA. For example, DMARD treatment, and anti-TNF-α targeting has several beneficial effects in RA, including suppressing inflammatory cytokine and chemokine production and anti-angiogenesis [3, 10, 12]. Thus, the role of inflammatory chemokines and cytokines in angiogenesis in RA is overlapping and may be useful for future targeting.

Conclusions

We have discussed the putative role of inflammatory chemokines, growth factors and cytokines in inflammation-associated angiogenesis. A number of CXC chemokines, maybe other chemokines and pro-inflammatory cytokines are involved in the angiogenic, as well as inflammatory events underlying the pathogenesis of arthritis. In addition, inflammatory angiogenesis research has important clinical implications. The assessment of synovial vascularisation and the synovial expression of some angiogenic factors may have prognostic role for the progression of RA, as well as other chronic inflammatory diseases. Moreover, anti-angiogenesis targeting using chemokine or cytokine inhibitors may control synovial inflammation and thus will benefit our patients.

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Modelling angiogenesis in inflammation

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Angiogenesis in inflammation

Angiogenesis is an integral component of chronic inflammatory lesions and is essential for tissue development and repair. The inhibition of this process is a target for the development of novel therapeutics against chronic inflammation, especially those diseases where angiogenic blood vessels feature prominently, such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD) and psoriasis. The development of these vessels is stimulated by factors produced within the inflammatory milieu and are derived from inflammatory cells, not least macrophages, which produce angiogenic factors under the hypoxic conditions found within these tissues. Macrophages for example have the extraordinary capacity to produce just about every angiogenic growth factor and cytokine known [1–3], such as tumour necrosis factor (TNF)-α, basic fibroblast growth factor (FGF-2), transforming growth factor (TGF)-β, angiotropin, prostaglandin (PG) E_2, granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, platelet-derived growth factor (PDGF), interleukin (IL)-6, vascular endothelial growth factor (VEGF), nitric oxide (NO), and angiotensin converting enzyme (ACE), but not all, angiogenin and platelet factor (PF) 4 being absent from their armamentarium. They do synthesise thrombospondin-1 (TP-1), which may be angiogenic or angiostatic depending on whether it is matrix bound or in the soluble or truncated form [4–6]. Thus, angiostatic factors may also be synthesised or elaborated, e.g., macrophage-derived enzymes such as metallo-elastase may mediate angiostatin release [7].

It is important that the therapeutic restriction of angiogenesis in chronic inflammation is not seen purely as a molecular tourniquet but as a disease-modifying treatment targeting the microvasculature. The arcades of blood vessels maintain disease chronicity not only through the supply of nutrients, but also the provision of cytokines such as IL-1, IL-6 and IL-8; growth factors such as PDGF; and mediators

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of vascular permeability such as the endothelins, PGI_2 and NO. These factors can be derived from endothelial cells (ECs), which also function to recruit leucocytes that subsequently release angiogenic factors, regulate plasma exudation with oedema and fibrin deposition (the first stage of angiogenesis) and blood flow, and act as antigen-presenting cells. Reactive oxygen species are another EC product, produced enzymatically by xanthine oxidase under hypoxic conditions. Therefore, angiogenesis and ECs [8] support chronic inflammation in a number of critical ways and the processes involved offer a plethora of opportunities as drug targets, each of which requires verification in models of inflammation and inflammatory disease [9].

When considering any experimental aspect of angiogenesis in inflammation, it is important that the methods for its assessment are considered in depth. This is a very difficult area, especially since inflammation has been regarded as a hindrance and confounding factor in the search for models of pure angiogenesis. Such pure systems of course have an equal relevance in predicting neovascular outcome in inflammatory disease as they do in oncology and have been reviewed elsewhere [2, 10–13]. The assessment of angiogenesis in inflammation *in vivo* is, however, problematic, especially when the aims are to produce a rapid direction for angiostatic chemistry. Care should be taken in inferring that disease modification in models of clinical disease is through angiostatic activity, knowledge of which has been derived from models of *in vitro* angiogenesis, or single stimulus *in vivo* models, unless the candidate is well characterised. This is due to the complexity of the inflammatory response. As an example, the anti-rheumatic action of the methionine aminopeptidase-2 inhibitor TNP40 (AGM1470) [14, 15] could equally be due to interference in T and B cell function [16–19], whereas the analogue PPI-2458 should be more selective [20], having been developed specifically as an MeA2I inhibitor as opposed to being a natural product. PPI-2458 in this report did not affect off-target immune cell functions and is thus selective.

Similarly, p38 inhibitors are angiostatic *in vivo* but due to the central role of the molecular target as in intracellular messenger, their disease-modifying mode of action cannot be said to be due to angiostasis. In the same vein, drugs that reduce inflammation will have profound actions on vascular tone, permeability and plasma exudation, whether they are anti-angiogenic or inhibitors of inflammatory mediators, as in the case with non-steroidal anti-inflammatory drugs (NSAIDs). If not taken into account, this could have misleading effects on certain systems that utilise intravascular tracers or blood flow to measure vascular volume. Thus, the specific assay of drugs for angiostatic activity in an inflammatory site is difficult to achieve *in vivo*.

The assay of angiogenesis in the inflammatory locus

There are four main methods that have been brought to bear on the problem: morphometric or histological analysis [21], blood flow through either laser or ultra-

sound Doppler flowmetry [22, 23] or the clearance of chemo- or radio-tracers [24, 25], and the determination of vascular volume through haemoglobin (Hb) content or vascular casting [26, 27]. All but histology depend on the presence of a discrete lesion either for injection of tracers or for later dissection.

Histological analysis

Histological analysis is definitive in providing a measure of vascular density [21]. It is painstaking and, as such, has often been used as confirmation of other more tractable techniques. It thus does not easily lend itself to drug screening. However, this is the method of choice for the analysis of human clinical samples as well as animal models of disease such as collagen-induced arthritis (CIA) and IBD [28].

Histological markers

Although there has been an International Consensus on the histological determination of angiogenesis in tumours [29, 30], this has not been regularly applied to inflammatory angiogenesis.

Capillaries can be seen in H&E-stained sections; those who have compared these with immunohistological methods will have seen the disparity in sheer numbers between the two methods. ECs display a wide variety of markers, some of which are considered selective. CD31 or lectins such as *Griffonia simplicifolia* lectin-1 (GSL-1) or *Lycopersicon esculentum* lectin are favoured markers, as well as von Willebrand Factor (factor VIII, vWF). The antigen CD34 is also an important co-antigen with CD31 for tumour vasculature visualisation. However, these are only as accurate as the selectivity of the antibodies, and indeed the distribution of the markers themselves.

The 130-kDa transmembrane glycoprotein of the immunoglobulin superfamily, CD31, is expressed at the endothelial intercellular junction and is widely used as a marker in tumour biology [31]. However, it is found on platelets, monocytes macrophages as well as neutrophils. vWF is found in Weibel-Pallade bodies where it is stored and secreted as ultra-large multimers, or is synthesised and secreted as low-molecular weight multimers. ECs secrete vWF in inflammation, which is measurable in blood, and are stimulated to do so by TNF and IL-8 [32], leading to the possibility that it may result in the underestimation of capillary numbers in the inflammatory locus. Interestingly, VEGF stimulates EC vWF Weibel-Pallade body exocytosis, but VEGF pretreatment inhibits this through the activation of endothelial nitric oxide synthase (eNOS) [33]. Thus, interventions that target these processes could interfere with this biomarker. CD31 on the other hand is widely considered constitutive in ECs.

CD31 is an important cell adhesion molecule for inflammatory cell migration through homotypic and heterotypic ligation to CD38 or integrins, and is found at the EC gap junctions. It is involved in both the induction of thrombus formation and cell transmigration in angiogenic ECs, and plays a role in angiogenesis [34–36] as well as a variety of other cellular functions based on its tyrosine kinase activity [37, 38]. CD31 is expressed in more than 90% of vascular tumours. In comparison, vWF is only expressed in 50–70%. While it is considered to be constitutive, the cytoplasmic tail of CD31 contains four endocytic sorting motifs and there are conditions where it can be internalised, destroyed or recycled and expressed. These are familiar phenomena in tumour cells [39], and may describe some of the difficulties others have had in detecting CD31 in tumours [31]. As regards inflammation, ECs incubated with TNF and interferon (IFN)-γ induce a redistribution of CD31 away from the EC gap junctions [40], and when combined [39, 41], degradation and synthesis inhibition. This turnover process could thus be sensitive to drugs, especially those that affect the Golgi, lysosomes, and cytoskeletal function as well as TNF-α and IFN-γ. CD31 can also be expressed in a wide variety of cells, including monocytes and histiocytes amongst others (see Tab. 1).

Unlike CD31, CD34 is not expressed by monocytes [42]. It is a highly glycosylated glycoprotein expressed by haematopoietic progenitor cells, endothelial precursor cells, ECs and, curiously, epithelial bulge cells [43]. It is not expressed by lymphoid vessels except high endothelial venules (HEVs), where it can bind L-selectin only at this site [44] probably due to variations in glycosylation patterns.

Few studies have compared the differential expression of CD34, CD31 and vWF in the normal vascular tree. ECs are heterogeneous for a variety of factors and function in an organ- and tree-dependent fashion, not least through the expression of adhesion molecules, chemokines and cytokines, but also tissue plasminogen activator (tPA) [45], ACE, fibronectin [46] and prostacyclin [47].

Those studies that have been carried out indicate homogeneous expression of CD31 in the human lung vascular tree [48, 49]. vWF on the other hand is expressed most strongly in veins, and progressively less so down the vascular tree with weakest staining at the level of the capillary with weak or even no staining [50, 51]. In the mouse [52], vWF antigen is detectable in the vascular tree in a fashion similar to that found by Pusztaszeri and colleagues in humans [49]. However, this is not reflected by vWF mRNA, which is expressed in abundant levels in lung capillaries, but not those in liver and kidney. This suggests a high turnover of vWF. vWF expression appears also to be influenced by the tissue micro-environment [53].

Pusztaszeri and co-workers [49] undertook a comparison of EC markers for their tissue distribution and down the vascular tree, including CD34, CD31 and vWF. Variations in the expression of both markers were found in both tissue and vessel types (summarised in Tab. 1). The new tumour vascular EC nuclear marker Fll-1 was also assessed, but stained all lymphocytes.

Table 1 - Staining of endothelial cells for CD31, vWF and Fli1-1 in adult human tissues (from Pusztaszeri et al. [49]).

Tissue	CD31	CD34	VWF
Kidney			
Glomeruli	+++	+++	0/+
Capillaries	++	+++	++
Venules	+++	+++	+++
Arteries	+++	+++	+++
Lung			
Capillaries	+++	+++	0/+
Arterioles	+++	+++	++
Venules	+++	+++	++
Veins	+++	+++	+++
Arteries	+++	+++	+++
Liver			
Periportal sinusoids	+++	+++	++
Centrolobular sinusoids	+++	0/+	++
Centrolobular veins	+++	+++	++
Portal venules	+++	+++	++
Arterioles	+++	+++	++
Spleen sinusoids	++	0	++
Capillaries	0	+++	0/+
Central arteries	+++	+++	++
Venules	+++	+++	++
Lymph nodes			
Capillaries	+++	+++	++
Marginal sinuses	+++	0/+	0/+
High endothelial venules	+++	+++	+++

Intensity of immunohistochemical staining: 0, absent; +, low; ++, medium; +++, high.

CD34 stained ECs throughout the tree and tissues. CD31 followed previous reports on the lung, with good staining throughout, but with a graded expression of vWF increasing up the tree from the capillary to the vein. A variety of staining patterns was seen in the kidney, with vWF having a focal distribution in fenestrated capillaries unlike CD31, which stained strongly throughout. Diffuse staining for CD31 and vWF was seen throughout the spleen, which was weak for vWF. In the liver, the patterns of vWF and CD31 were similar, and in the skin all ECs stained

with each marker. Of greater interest here, both vWF and CD31 stained HEVs in lymph nodes, but sinusoidal endothelium was negative for vWF. CD34 is recognised not to be expressed in lymph nodes, except in lymph node HEVs. Non-EC staining for vWF has not been reported, except in areas of large vessel walls depleted of ECs (thus not CD31 positive), which the authors noted could be consistent with sub-endothelial matrix-bound vWF [54]. Non-EC staining with CD31 was also minor compared to CD34 and Fll-1. CD31 stained lymph node histiocytes and alveolar macrophage cell membranes. Other cells such as neutrophils and macrophages express CD31. The macrophage staining of CD31 is considered to be a concern when assessing ECs in poorly differentiated tumours [55].

Under inflammatory conditions, vWF and CD31 behave less consistently probably depending on the type of inflammation model. LPS and TNF administration increase circulating vWF in mice [52], as does LPS in humans [56–59], and stimulates vWF release from ECs *in vitro* [59]. The action of LPS in mice is paradoxically accompanied by a concomitant reduction in CD31 mRNA expression in most tissues, except for heart kidney and gut.

It should be noted that under a hostile inflammatory locus EC function may be compromised, and this may be reflected in the expression of these markers. In glomerulonephritis both vWF and CD31 stain all vessels, but sometimes with a decreased intensity [60], where this is taken as an indication of reduced EC functionality and compromise, as in the case of IgA glomerular nephropathy [61]. Indeed, in experimental models of glomerulonephritis, CD31 expression is used to show regenerative capacity of capillaries [62, 63], and its loss as a failure in regeneration in progressive disease. In these studies CD31 staining is not seen in areas of macrophage infiltration, thus reflecting EC damage through macrophage activation.

In animal models of inflammatory disease, vWF and CD31 are used to illustrate and quantify angiogenesis. Angiogenesis in the synovium of rats with CIA can be visualised using anti-vWF [64], and quantified through counting the number of vessels in three 100× fields. In this study kallistatin gene transfer inhibited joint erosion, neutrophil accumulation, vWF blood vessel density, TNF and IL-1 synthesis, and reduced angiogenic factors in joint washouts. Tsai and co-workers [65] have used synovial vWF expression to illustrate the inhibition of angiogensis using the VEGF-binding activity of nanogold coupled with disease-modifying activity. These authors averaged the microvessel density from five 400× fields. In addition to the histological visualisation of vessels through CD31 in murine CIA, the assay of joint vWF yields quantitative data [66]. Rat synovium in an adjuvant-induced arthritis model also expresses vWF [67].

CD31 has also been utilised in rat adjuvant-induced arthritis. Devesa and co-workers [68] report a qualitative reduction in CD31-positive vessels in rats treated with the HO-1 inhibitor, SnPP. The illustrations show selective staining for vessels, with very few macrophages, if any, staining, perhaps due to the neutrophil dominance of this disease. Alternatively, Safronin-O staining has been used in peptidogly-

can-polysaccharide (PG-PS)-induced arthritis in rats in conjunction with Bioquant software. In this instance only cross-sectioned vessels with a defined endothelium and lumen were measured [69], showing that a TP-1-derived peptide can reduce both angiogenesis and joint pathology.

A comparative study of a variety of markers for ECs in human inflammatory disease, RA, osteoarthritis (OA), and Crohn's disease has been reported by Middleton and co-workers [70], amongst them CD31 and vWF. Unlike MECA-79, DARC, CD34, CD146 and CD105, CD31 and vWF stained for ECs at all levels of the vascular tree in RA and OA synovium, as well as Crohn's disease. vWF specifically stained ECs, but CD31 stained subintimal fibroblasts, macrophages and lymphocytes in RA synovium, whereas mononuclear cells were also stained in Crohn's disease.

Angiogenesis in Crohn's disease was definitively described using an antibody cocktail against vWF and CD31 [71]. In experimental colitis, counting CD31-expressing cells as a ratio of total nuclei gives a good end point of 'angiogenic index' in both dextran sulphate and $CD4^{+}CD45RB^{high}$ T cell transfer murine models of IBD. This index was compared by Chidlow and co-workers [28] to *L. esculentum* lectin staining, and both markers co-localised, except in a few extraneous cells expressing CD31. The angiogenic indices gave the same increases in index for each disease, although the overall angiogenic indices for CD31 were higher than those for the lectin. In this model, the development of the vasculature precedes the histopathology, reaching its maximum coincident with the maximum histopathological score [28]. At this maximum, the angiogenic index correlates well with the histopathological score in both the dextran sulphate and $CD4^{+}CD45RB^{high}$ T cell transfer IBD models. The use of CD34 and ICAM-1 in trinitrobenzene sulfonic acid (TNBS)-induced IBD in bone marrow-transplanted mice has shown that marrow-derived progenitor cells play an active role in the inflammatory angiogenesis [72].

L. esculentum lectin and GSL-1 bind with different vascular binding patterns, with *L. esculentum* having a preference for microvessels [73]. GSL-1 binds α-D-galactosyl residues on ECs [74], now defined as the xenograft Galα(1–3)Gal antigen [75]. It correlates well with the EC staining for CD31 in the murine chronic granulomatous air pouch [76].

From these studies, it is difficult to make a definitive statement as to the 'best' histological marker for angiogenesis in inflammation. Perhaps a lesson could be learned from tumour angiogenesis, where the Second International Consensus (SIC) on the methodology and criteria of evaluation of angiogenesis quantification in solid human tumours lists a variety of procedures for the clinical evaluation of angiogenesis. These use: the Chalkley grid point overlap quantification method with CD34, a method that is used to quantify the relative vessel density to determine breast carcinoma survival; the pericyte coverage index with CD34 or CD31 double stained with alpha smooth muscle actin, which can be used for the assessment of VEGF-based therapies; and EC proliferation fraction through double immunostain-

ing with CD31 and the proliferation antigen Ki67, used for assaying angiogenesis inhibition in activated or proliferating ECs.

However, in the inflammatory locus, we have seen here that the expression of both CD31 and vWF are affected by inflammatory cytokines and mediators. CD31, depleted through either reuptake, synthesis inhibition, or even EC cell death, and vWF may suffer from secretion and synthesis inhibition at the inflammatory site. While vWF is rarely found to be expressed on other cells, CD31 can certainly be expressed by a variety of myeloid cells. CD31 can be used as an index of angiogenesis in tumour biology in the presence of high numbers of macrophages. In several studies the staining of CD31 appears selective. In any event, non-EC staining is often clear and could be taken into consideration through the counting of patent capillaries only. The major differences in the two fields is the absence of vWF in the tumour SIC, and the absence of CD34 in models of inflammation.

Definitive work along the lines of the SIC [30] has been reported by Walsh and colleagues in 2003 [77]. CD34, with an index of cell proliferation (proliferating cell nuclear antigen, PCNA), and comparison to smooth muscle α-actin in fibrosing alveolitis provides clear and definitive proof of the role of angiogenic capillaries in the fibrous tissue, and the determination of EC fraction, as well as distances from the alveolar space. Interestingly, in this study showing that while the fibrotic tissue contains angiogenic vessels, the EC fraction is significantly less than that of the healthy tissue. This is very similar to the report of Stevens and colleagues [21] comparing normal and rheumatoid synovium, and agrees with the notion that angiogenesis in arthritis may not be sufficient to maintain normal oxygen tension [78]. Walsh and co-workers [79] have also marked vascular endothelium, proliferating endothelium, and macrophages in human OA synovium, achieved through the use of CD34, CD31/Ki67 (marking EC proliferation), and CD14, respectively. This study demonstrated that OA synovial angiogenesis is related to the synovitis, and not other manifestations of OA joint disease. CD31 ECs were interactively identified with image analysis. In addition, CD34 identified the vasculature of the osteochondral junction [79]. These studies could be considered definitive, and could thus point the way in the determination of angiogenesis in animal models.

The best course of action is thus to properly characterise the vessel staining characteristics in each type inflammation lesion investigated in the model species, with respect to lectin, vWF, CD31 and CD34, to determine the overlap of markers and also to develop the protocols to enhance EC staining relative to non-EC staining. The section thickness should be considered to highlight vessel cross-section area and patency (3 μm) or thicker sections for visualising capillary length and tortuosity (15 μm). The inclusion or exclusion of patent vessels will highlight different phases of the angiogenic process. The co-localisation of endothelial markers with indices of proliferation (Ki67, PCNA) will give an accurate representation of the active angiogenic process, and patent vessels along with the use of smooth muscle α-actin will mark their maturation, and give a historical record of recent angiogenesis.

Vascular flow, volume, and density determination in models of inflammation as measures of angiogenesis

From the previous section, it can be seen that definitive alterations in angiogenesis can be determined histologically; however, several issues arise from this. For angiogenesis screening histology remains labour intensive and requires histological laboratory support. In addition, assessment often centres on the most angiogenic regions, or 'hotspots', whereas in inflammation the angiogenic component of the whole tissue would be desirable. The organisation of blood vessels in various types of models of inflammatory varies.

In the sponge granuloma, for example, CD31 immunohistology reveals blood vessels invading the sponge matrix from the subcutaneous tissue this is not uniform;, there are clusters of capillaries (Fig. 1c). The mBSA delayed-type hypersensitivity (DTH) granuloma [80] exhibits arcades of vessels stretching into the granuloma, and the murine chronic granulomatous air pouch possess a highly active angiogenic front fed by arcades of vessels derived from the subcutaneous vasculature (Fig. 1a, b, d, and see below). For comparison, the murine colorectal adenocarcinoma Colon-26, develops an even spread of vessels that can be easily counted (Fig. 1e).

Thus, a variety of techniques have been developed that utilise assessments of vascular volume, or blood flow. These are aimed at permitting the use of larger groups of animals for drug efficacy testing and the direction of medicinal chemistry. In addition, they may be considered secondary preclinical screens in that they are designed to determine changes in inflammatory angiogenesis as the primary end point, and inflammation reduction as a secondary end point. Tertiary preclinical screens would utilise disease models featuring angiogenesis, and the prime end points for these trial would be inhibition of disease as well as reduced blood vessel development.

Doppler flowmetry

Laser Doppler flowmetry, using a surface probe for example, has been successfully utilised in DTH granulomas and cotton pellets in rats [22]. This has the potential advantage of linear comparison of developing lesions within the same animals. However, peripheral vasodilatation is required through warming the animals. The results need to be carefully controlled for cutaneous blood flow, which is affected by arterio-venous shunting. The positioning and pressure of the probe on the skin is so critical that comparative studies are in practice very demanding. Superficial probes will also only measure the outer perimeter of the lesion, the limit of measurement being only around 1–3 mm into the tissue, unless power ultrasound Doppler flowmetry is used. The deeper zones of the lesion should not be measured through subcutaneous probes, as these require long periods of stabilisation, all under anaesthesia,

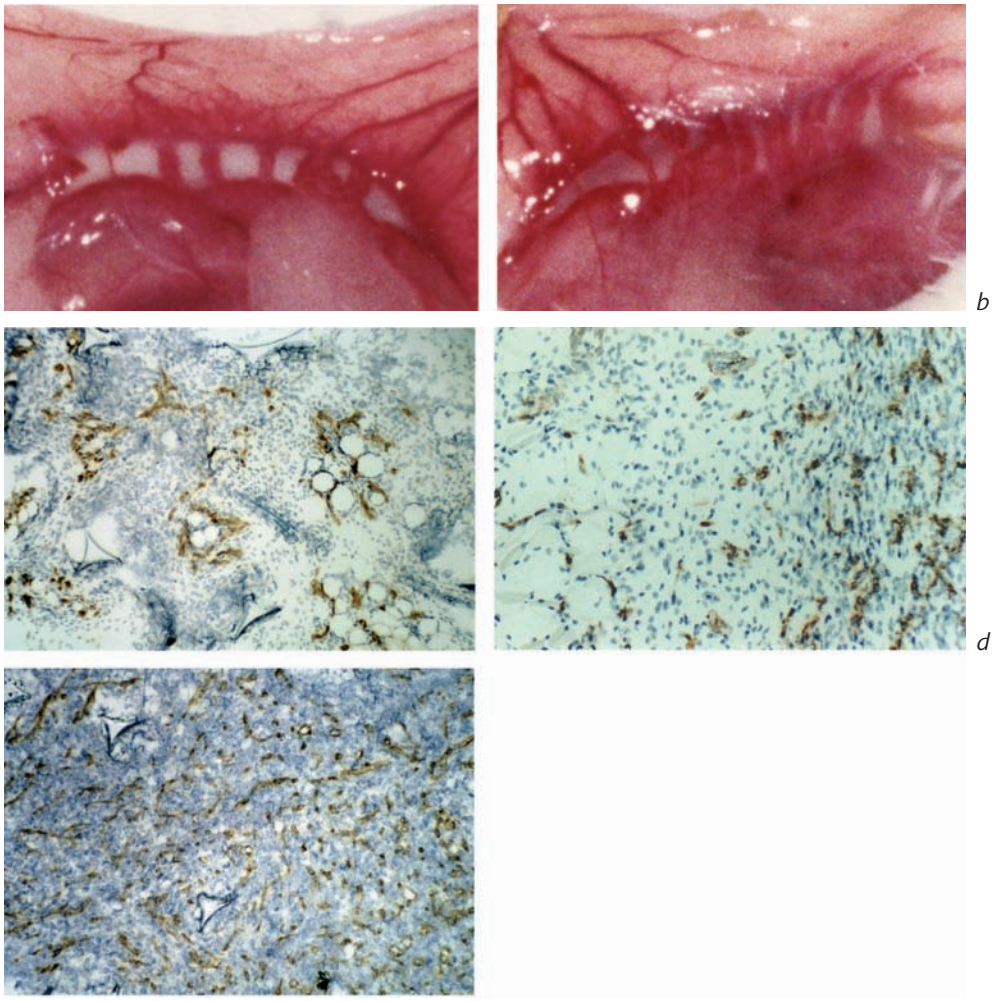


Figure 1
Modelling angiogenesis in inflammation. The murine chronic granulomatous air pouch capillary network visualised by the carmine vascular cast at 4 days (a) and 6 days (b). The distribution of capillaries within tumour and different inflammatory tissues visualised by immunostaining for the endothelial cell marker CD31 in: (c) murine day 8 sponge granuloma, (d) murine day 7 chronic granulomatous air pouch, and (e) murine day 8 colon-26 adenocarcinoma. Sections cut at 15 μm to reveal tortuosity.

to overcome the effects of fluid flow equilibration after surgery and implantation. This means that is is difficult to use the number of animals that are required for dose-response and comparative drug studies.

Power ultrasound Doppler flowmetry is a new and powerful tool that is being applied to the small joints of RA patients detecting enhanced vascular flow in RA wrist and metacarpo-phalangeal (MCP) joints [81–84]. This is applied to detect areas of high flow. This has been applied recently with success to the murine CIA providing a translational end point between the preclinical disease model and human RA [23]. Under these circumstances, the end points can be characterised fully to determine differences between acute pharmacological manipulation of vascular flow and chronic disease-modifying anti-angiogenic activity.

Vascular volume

Three other methodologies are used to determine the size of the vascular bed in inflammatory lesions. These are commonly based on vascular tracer clearance rates for ^{133}Xe [24] or fluorescein dye [25], Hb content [85], or the creation of vascular casts [26, 27] within dissectible lesions. Each of these concepts has been compared by Fan and co-workers in one study using the sponge granuloma, with a high correlation [24, 86]. Each of these requires a dissectible lesion, and the clearance methods are restricted to the sponge granuloma. ^{133}Xe clearance is related to the vascularity of the tissue, and could be construed as being an index of the granuloma vascular volume. It is accepted that modulation of these indices are accompanied by at least a qualitative histological assessment of vascular density or some other illustration of the vasculature such as whole vascular casts [26].

Vascular tone, plasma exudation, and surrogate indices of angiogenesis

Pharmacological interference with vascular tone and plasma exudation on inflammatory tissue indices of angiogenesis need to be considered. End points that rely on *in vivo* vascular volume, blood content through Hb, blood flow or clearance rates can all respond to acute changes in vascular tone and plasma exudation. This is especially true in the inflammatory locus, where inflammatory mediators have profound and interactive actions concurrent with the inflammation. Indomethacin for example inhibits ^{133}Xe clearance from rheumatoid joints, while not affecting clinical features [87], meaning that the effect is probably an acute action on the vasculature. Comparison of the tumour neovasculature to inflammation vasculature utilising ^{133}Xe clearance demonstrates that the tumour and sponge granuloma vasculature are different [88, 89]. The granuloma vasculature reacts with prolonged clearance of ^{133}Xe to the acute administration of vasoconstrictors including of adrenaline, 5-hydroxytryptamine (5-HT), angiotensin II (AII), platelet-activating factor (PAF), and endothelin, whereas the tumour-bearing sponges lose reactivity as the tumour develops. This does not appear to be due to the absence of contractile elements since

perivascular actin is present to an equal degree in both. This would apply to all such methods, such as the clearance of fluorescein [25]. Interestingly, endothelin has vasoconstrictor actions when administered within the sponge granuloma and when administered subcutaneously above it. However, histamine is only active if given subcutaneously [25]. This shows that the tone of the subcutaneous vessels is important for the supply of blood to the lesion. This, coupled with the observations that cutaneous vasodilatation is required for adequate granuloma perfusion, as assessed by laser Doppler flowmetry [22], led to the use of thermal vasodilatation coupled with the carmine method [26]. Comparison with Evan's blue extravasation showed that the chronic granulomatous air pouch responds to intra-pouch histamine and endothelin to increase and reduce plasma exudation, respectively. Inhibition of PG synthesis by oral indomethacin also inhibits plasma exudation. Carmine vascular casts are not susceptible to these acute changes, and thus reflect vascular volume and density. In fact, the chronic administration of indomethacin and some traditional cyclooxygenase (Cox) inhibitors (Tab. 2, [90]) reduces the vascular volume as assessed by this method. Thus, familiarity with the vascular dynamics of the method of choice, coupled with a chronic dosing regime with an adequate washout period should ensure accurate determinations of vascular volume independent of acute vasoreactivity. This being said, the research into the vasoreactivity of the neovasculature in inflammation and tumour biology is very well served by these vascular clearance systems [25, 88, 89, 91].

Carmine gelatin vascular casting

We chose the carmine/gelatin vascular cast method originally pioneered by Kimura and coworkers [27, 92] as the method of choice following its modification and characterisation at Hoechst UK (Seed MP, Rising TJ, Halford J, unpublished data, 1986). A non-histological method was required since the aim was to investigate the cotton pellet/cartilage co-implant model [93]. The cotton pellet is very demanding to section for histology. The carmine cast method was modified [26] to prevent loss of sensitivity through dye bleaching during tissue maceration, to incorporate peripheral vasodilatation similar to that found by Orlandi and coworkers [22], and calculate vascular density as opposed to volume. The dry mass was used to assess granuloma size since wet mass can reflect alterations in fluid volume through inflammatory exudation. On a practical note, warming the animals prior to injection of the carmine/gelatin dye/casting mixture provides the most reproducible results, while vasodilator agents such as hydralazine perform poorly due to poor tissue perfusion (Freemantle CN, Brown J, Seed MP, 1986, unpublished data). Comparison of the ^{133}Xe and carmine methods in the sponge granuloma have given a correlation coefficient of 0.97 for the respective indices of vascular volume, correlating well with Fan and coworkers [24, 86]. If clearance is expressed per unit wet mass, the two

Table 2 – The development of granulomatous tissue, vascular content (mg dye), and vascular index (VI) (μg/mg, in mice treated with anti-rheumatic drugs); n=*8 per group,* **p<*0.01,* *p<*0.05 Mann-Whitney U test compared to vehicle control. Chronic granulomatous air pouches were induced by the s.c. injection of 3 ml air into anæsthetised mice (30 ± 2 g, Tuck) and 0.5 ml Freund's complete adjuvant with 0.1% croton oil 24 h later. Mice were dosed p.o. for 6 days with nonsteroidal (NSAID), steroidal and slow-acting anti-rheumatic drugs. Vascular content was assessed by the formation of a vascular cast [26, 76] by i.v. injection of 1 ml 10% carmine red in 5% gelatin at 40°C, and chilling the carcasses. The dissected tissue was dried, and papain digested; the dye was dissolved at alkaline pH, and read at 490 nm after centrifugation and filtration. Results are expressed as either mg dye content per sample or the VI as μg dye/mg dry weight of tissue.*

Treatment (mg/kg)	Dry mass (mg)	Dye (mg)	VI (μg/mg)	Drug (mg/kg)	Dry mass (mg)	Dye (mg)	VI (μg/mg)
p.o. vehicle	123 ± 11.0	2.48±0.24	20.2±0.6	Chloroquine (50)	124± 7.0	0.84±0.06**	6.6±0.3**
i.m. vehicle	122±10.0	2.39±0.22	19.5±0.7	Aurothiomalate (20, i.m.)	108± 8.0*	0.89±0.13**	8.4±5.5**
Indomethacin (1)	149±16.0**	1.77±0.26**	11.9±0.9**				
Ibuprofen (30)	142±16.0	1.33±0.09**	9.4±0.3**	Auranofin (20)	125± 7.0	0.66±0.07**	5.0±2.5**
Piroxicam (2)	130±12.0	2.04±0.11	15.7±1.2	Cyclophosphamide (10)	118± 9.0	0.81±0.06**	6.9±0.5**
Dexamethasone (0.5)	53± 8.0**	1.16±0.15**	23.7±1.1**	Azathioprine (30)	137±10.0*	1.72±0.08**	11.7±0.5**
Prednisolone (10)	102±8.0*	1.10±0.15**	10.3±0.8**	Methotrexate (0.6)	109± 7.0	0.61±0.09**	5.8±0.3**
D-Penicillamine (100)	107±5.0*	0.74±0.13**	7.1±0.7**	Levamisole (50)	117± 6.0	1.52±0.39*	12.7±0.3**

Those agents that reduce cell proliferation, reduced the VI, as did the NSAIDs, except for piroxicam. The gold-containing agents reduced the VI significantly as did D*-penicillamine, in keeping with their* in vitro *actions, inhibition of endothelial cell proliferation* in vitro *[224]. Prednisolone was also effective, but dexamethasone reduced granuloma formation to such an extent that it resulted in an increase in the VI. The activity of the NSAIDs could reflect the angiogenic activity of* PGE_2 *[166].*

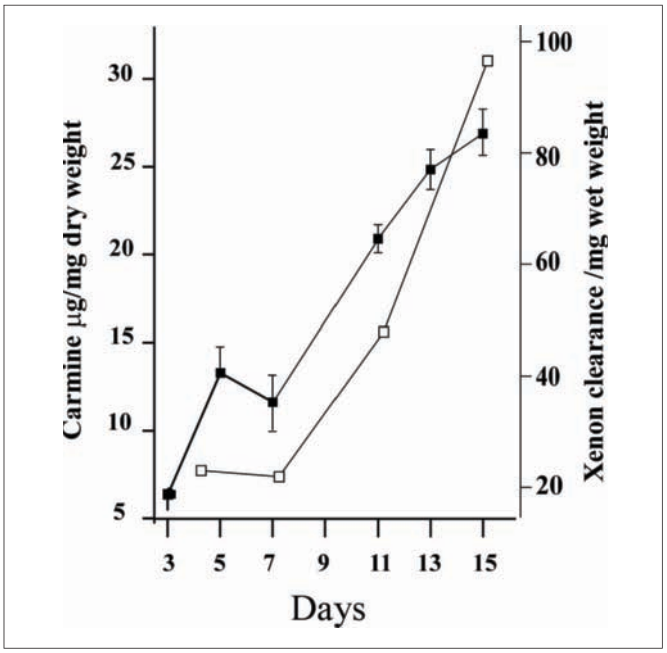


Figure 2
Comparison of two methods for assessing angiogenesis in inflammation from the tissue vascular volume by assessed by carmine/gelatin vascular cast (closed squares) or ^{133}Xe clearance (open squares) expressed as a function of tissue dry and wet mass, respectively.

methods follow similar paths especially considering that the data are gleaned from two different laboratories (Fan and Seed, Fig. 2). The carmine method has been used in a variety of settings in addition to the murine chronic granulomatous air pouch, including gastric ulcer healing [94], rat croton oil [95] or carageenan [96] air pouches, and rabbit joint structures [97].

The carmine method has thus enabled the comparison of different angiogenic responses in different loci. The sponge granuloma appears to develop in a slow and consistent fashion with a correspondingly persistent development of vascular density (Fig. 3a). On the other hand, the development of the murine chronic granulomatous air pouch is very different, and corresponds to the histology. The induction of the highly angiogenic front is co-incident with a peak of vascular density at day 5 (Fig. 3b). Following this as the tissue develops, 2 days later these vessels extend and the vascular index is reduced and converts to the chronic phase by day 14. The method thus takes into account the whole tissue, i.e. the angiogenic, maturing and mature vessels, as opposed to selected sections of it. As an example of solid tumour development, the murine Colon-26 colorectal adenocarcinoma implanted into the sponge

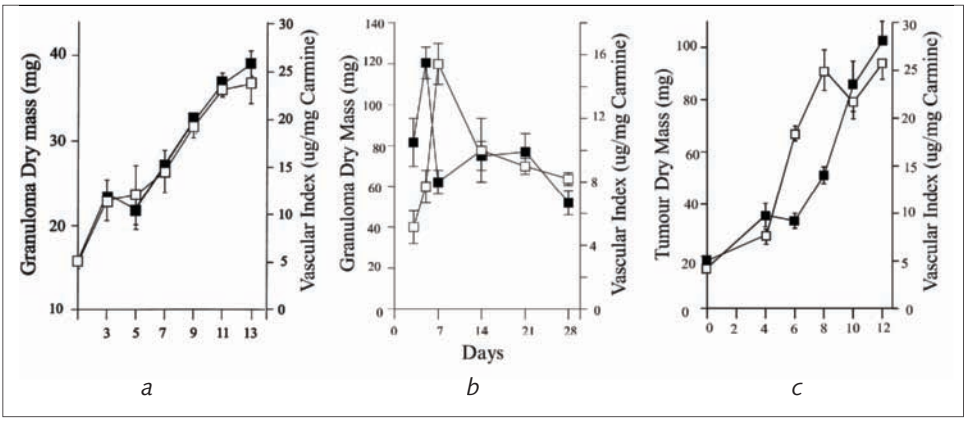


Figure 3
The development of the vasculature as assessed by carmine/gelatin vascular casting by different subcutaneous reactions in the mouse. (a) Sponge granuloma (Brown J, Seed M); (b) murine chronic granulomatous air pouch [26]; (c) murine colon-26 colorectal carcinoma [98]. Carmine in gelatin is administered i.v. under terminal anaesthesia, followed by dissection of the tissue, drying, and maceration [27, 92, 155]. The carmine is then assayed spectrophotometrically and expressed as µg carmine/mg tissue dry mass.

demonstrates a clear 'angiogenic switch' (Fig. 3c). In this case, the cells are injected into a 3-day sponge granuloma, and their development is retarded until the vascular volume reaches a critical peak at day 8; the tumour then develops rapidly over the next 2 days. The tumour apoptotic index, as assessed by DNA-end labelled cells is $0.12 \pm 0.02\%$ ($n = 4$) at day 6 and switches dramatically to $0.05 \pm 0.01\%$ ($n = 4$) [98] during the angiogenic phase. The ^{133}Xe method demonstrated a clear reduction in vascular clearance at the later stages of the Colon-26 tumour development, related to tumour necrosis, and in Figure 3c the plateau of the vascular bed is matched with histological evidence of zones of tumour necrosis.

Haemoglobin

Once a simple ELISA to Hb became available, Hb content was used widely as a measure of vascular volume in tumour biology, and Andrade and co-workers [85] have characterised this method in the inflammatory locus, and correlated it with fluorescein clearance, focusing on the sponge granuloma. Erythrocytes have the advantage that they are confined to the vascular compartment; a disadvantage could be that acute changes in vascular tone could skew the results. If treatments are liable to reduce the exudate content of the tissue, and thus wet mass, it is conceivable that

this index may give an underestimate of angiogenesis inhibition, but this has never been shown. This method has the same advantages as the carmine method, permitting its use in a dissectible lesion but with the added advantage of not requiring a licensable procedure at the end of the study. It correlates also well with ^{133}Xe, carmine vascular cast, and histological counting of vessels [24]. It thus assesses the vascular volume of the tissue, expressed as units of Hb/unit tissue mass.

Testing inflammatory angiogenesis as a target for the treatment of chronic granulomatous diseases

A variety of inflammatory models and diseases are, therefore, pliable for the measurement of inflammatory angiogenesis by a variety of tried and tested means. These have been utilised in an equally imaginative fashion to determine the utility of a wide variety of potential angiostatic drugs and their targets.

The cotton pellet granuloma

Following the discovery that rheumatoid synovium, while appearing hyper-vascular, is in fact hypoxic with a low vascular density [99], it was not at all clear whether the inhibition of angiogenesis would be beneficial or detrimental. Early studies with tumours showed that angiostatic therapy may lead to tumour necrosis and the subsequent death of the host [100]. There was no evidence even that such inhibition would be disease modifying. There was also the problem of which therapies to use to test specificity for angiogenesis inhibition, while not modulating immunity or inflammation. The closest agents to fit this bill were the angiostatic steroids with or without heparin co-administration [101]. These were assumed from early steroid metabolism studies to be devoid of classical steroid activities. Such agents have been shown to not bind to mineralocorticoid, glucocorticoid, progesterone or oestrogen receptors [102]. They include tetrahydrocortisol, tetrahydrocortexolone, medroxyprogesterone, and cortisone in combination with heparin.

Initial studies with the cotton pellet granuloma and DTH granulomas as well as the sponge granuloma indicated that the development of granulomatous inflammation may be reduced by the use of angiostatic steroids [22, 103]. As with other studies in the tumour field, the source of heparin proved to be essential in promoting the angiostatic activity of low-dose cortisone (1 mg/kg). This dose is a sub-anti-inflammatory dose of cortisone in mice and rats, and, with the use of Monoparin, results in an inhibition of granulomatous tissue angiogenesis [26]. Inhibition is also seen with tetrahydrocortisol and methyl progesterone but in the absence of heparin [26]. These studies also showed that heparin alone can induce granulomatous tissue angiogenesis in a dose-related fashion. The sponge granuloma is also sus-

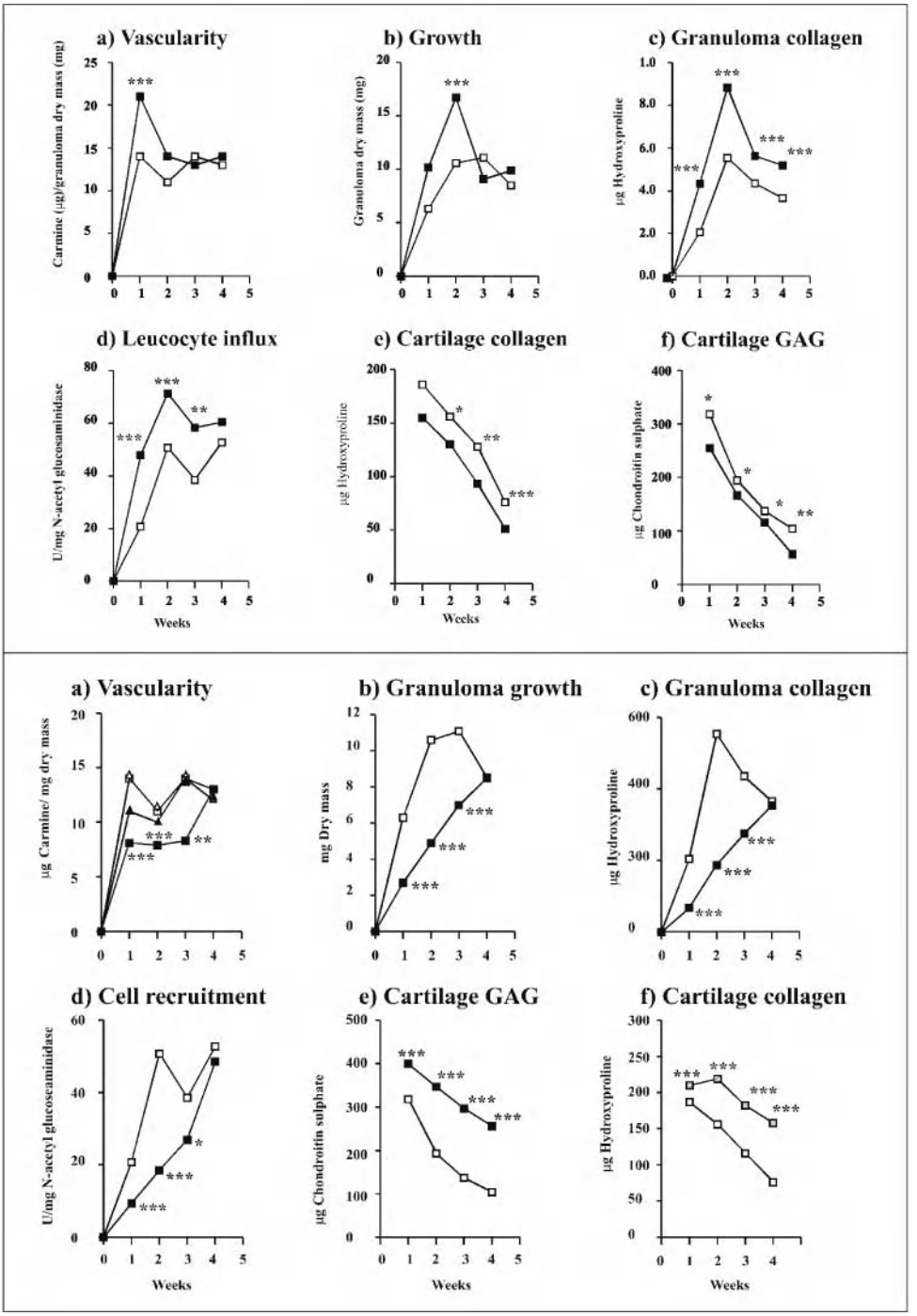
ceptible to angiogenesis inhibition by angiostatic steroids, including U24067 and tetrahydrocortexolone [104]. There is some structural selectivity here since both medroxyprogesterone and tetrahydrocortisone appear ineffective in the absence of heparin against sponge granuloma and chronic granulomatous tissue, respectively. The specificity of these effects is illustrated by the observation that angiostatic steroids do not modulate granuloma IL-1 and TNF-α synthesis, unlike the potent anti-inflammatory steroid dexamethasone that does [104].

What of the effects of angiogenesis modulation on the outcome of chronic inflammatory disease – tissue destruction? In these studies several parameters were measured to assess the effect of angiogenesis in pannus development and its destructive powers. The cotton pellet granuloma/cartilage co-implant model was used to assess a wide variety of parameters within the dissectible lesion, such as those for tissue growth, matrix deposition, cartilage viability and destruction, and inflammatory cell recruitment, as well as carmine vascular casting to assess the angiogenic component [105]. Rat femoral head cartilage was wrapped in sterile dental cotton and implanted subcutaneously in mice, resulting in granulomatous inflammation and cartilage destruction, concomitant with cytokine synthesis [106] as well as matrix metalloproteinase (MMP), elastase and cathepsin-G release [106]. Figure 4 shows the effect of the stimulation of angiogenesis (3000 U Monoparin p.o./day) as well as its inhibition (1 mg/kg cortisone s.c. plus Monoparin 1000 U/day p.o.). Cell recruitment assessed as N-acetylglucosaminidase activity was potentiated and inhibited, respectively, and in tandem with this, granulomatous tissue development was modulated. Cartilage catabolism was increased with angiogenesis stimulation, but was reduced in concert with angiogenesis inhibition, in fact almost arrested.

A modern equivalent of the angiostatic steroid, the cortisene steroid anecortave (AL-3789), is in clinical trial for diabetic retinopathy [107]. It was developed through screening retinal angiogenesis inhibition and iteratively screening out the anti-inflammatory glucocorticoid activity using carageenan paw oedema, LPS-induced uveitis in rabbits and rats, as well as *in vitro* assays of LPS-stimulated tPA-differentiated U937 macrophage cytokine synthesis [107–109].

Sponge granuloma

The sponge granuloma is used widely as a model of wound healing, for the testing of agents for wound healing, and of fibrovascular development. In the inflammation field, it has been used intensively as a test bed for the study of angiogenesis in chronic inflammation, especially the action of pro-angiogenic factors, such as vasoactive intestinal peptide (VIP), the endothelins, AII, PAF, IL-1, bradykinin, VEGF, substance P, TNF-α, IL-1, and CXCL1-3/KC and CCL2/JE chemokines [24, 91, 110–115]. IL-6 does not appear to modulate sponge granuloma angiogenesis [116]. The angiostatic steroids were the first agents used to establish the importance of


a) Vascularity
Carmine (μg)/granuloma dry mass (mg)
b) Growth
Granuloma dry mass (mg)
c) Granuloma collagen
μg Hydroxyproline
d) Leucocyte influx
U/mg N-acetyl glucosaminidase
e) Cartilage collagen
μg Hydroxyproline
f) Cartilage GAG
μg Chondroitin sulphate
Weeks
a) Vascularity
μg Carmine/ mg dry mass
b) Granuloma growth
mg Dry mass
c) Granuloma collagen
μg Hydroxyproline
d) Cell recruitment
U/mg N-acetyl glucosaminidase
e) Cartilage GAG
μg Chondroitin sulphate
f) Cartilage collagen
μg Hydroxyproline
Weeks

angiogenesis in the development of the granuloma, as they have in the other models mentioned here.

The sponge granuloma system is affected by the classical angiogenesis inhibitors. The angiostatic steroids U-24067 and tetrahydro-S, unlike dexamethasone, were effective in reducing ^{133}Xe clearance, without inducing the glucocorticoid receptor-mediated changes of thymic involution or TNF and IL-6 synthesis inhibition, which are characteristic of glucocortoid receptor activation [104]. In addition, sponge granuloma angiogenesis is inhibited by TP-1, as is the angiogenesis stimulated by VEGF [117]. TP-1 induces EC apoptosis through the induction of caspase-1 *in vivo*. The other commonly tested angiostatic agent, thalidomide, inhibits guinea pig sponge angiogenesis [118], but enhances foreign body multinucleated giant cells. In contrast, Andrade and coworkers [119] reported a reduction in the fibrovascular tissue concomitant with the suppression of angiogenic fluorescein clearance and granuloma Hb content at 100 mg/kg. Thalidomide is known to inhibit both TNF synthesis [120] and action [121], which could explain its activity in the inflammatory locus, unless shown otherwise.

A role for TNF receptor 1 (TNFR1) in inflammatory angiogenesis is an interesting concept. Barcelos and coworkers [122] used the Hb content of the sponge granuloma to differentiate angiogenesis from leucocyte infiltration. In this study TNFR1-knockout mice had an impaired ability to develop an angiogenic response, but inflammatory cell influx still occurred. This means that the anti-angiogenic actions of TNF suppression can be dislocated from the cellular effects seen in immune-mediated models of inflammatory disease by TNF inhibition.

There is some evidence that activation of the angiotensin AT1 receptor promotes angiogenesis, while AT2 mediates angiogenesis inhibition [123, 124]. However, the role of AII in inflammation appears different. AII modulates angiogenesis in the

Figure 4
The effect of angiogenesis stimulation (panel 1) and inhibition (panel 2, adapted from [105]) on the murine cotton/cartilage granuloma co-implant model of inflammatory cartilage erosion. Angiogenesis was stimulated by treatment with 3000 U Monoparin/day p.o. and its inhibition with low-dose cortisone (1 mg/kg/day s.c.) with 1000 U/day Monoparin p.o. (see [26]). Vascularity of the granulomas was assessed using the carmine/gelatin vascular method [26], granuloma growth by tissue dry mass, collagen assessed by the assay of hydroxyproline, leucocyte influx by N-*acetyl-glucosaminidase, cartilage glycosaminoglycan (GAG), by the method of Farndale et al. [223]. Open symbols denote vehicle-treated controls, and closed are the respective angiogenic/angiostatic therapy. In panel 2, controls are vehicle (water) alone, Monoparin at 1000 U/day p.o. alone or 1 mg/kg/day cortisone alone. In each case, except for panel 2a, the data overlap and are not shown for clarity. In panel 2a, open squares denote p.o. water, open triangles heparin 1000 U/day p.o., closed triangles cortisone 1 mg/kg/day s.c., and closed squares cortisone/heparin angiostatic combination. All points are* n=*8–10, significance calculated by Kruskal-Wallis multicomparison test.*

inflammatory locus, being pro-angiogenic in the 7-day murine sponge as assessed by ^{133}Xe clearance and histology [125], and Hb content and fluorescein clearance [126], as well as in the rat sponge as assessed by ^{133}Xe clearance [127]. Fibrovascular growth is also accelerated. This action is antagonised by the selective AT1 receptor antagonists losartan and DuP 532, but not the AT2-selective antagonist PD123319. AT2 stimulation with CGP42112A is ineffective, thus AII acts *via* the AT1 receptor to induce angiogenesis in the rat sponge granuloma. Interestingly, angiotensin I itself and a variety of ACE inhibitors had no action on the day 8 sponge angiogenesis [127]. However, ACE expression appears later in the development of the fibrovascular tissue, at day 14. AT1 expression appears within the first week, followed by AT2 expression as the microvasculature matures [127]. ACE and AT1 are expressed in RA synovium [128, 129]. In contrast, the septapeptide ANG1-7 is anti-angiogenic. This appears to show a dual role of the angiotensin receptors since the effect of ANG1-7 is mediated *via* the stimulation of angiotensin-I and –II receptors, its actions being inhibited by the receptor antagonist, A-779 [D-Ala(7)-ANG-(1-7)] [130]. In this study, it was demonstrated that this antagonistic effect could be mediated by NO synthesis.

So, in the absence of ACE in the first 2 weeks of the granuloma, AII release could be *via* enzymes other than ACE, such as chymase. Chymase is derived from mast cells [131] and has a direct role in sponge granuloma angiogenesis. AII is thus part of a cascade involving its release by chymase, its action on A-1 and A-2 receptors, as well as the involvement in the secretion of VEGF [132].

Anti-VEGF injected into the sponge inhibits the development of H&E-stained neo-vessels [133] and sponge Hb content. Being a heparin-binding protein, VEGF-induced ^{133}Xe clearance is inhibited by suramin [112, 134]. Between the VEGF isoforms VEGFA ($VEGF_{165}$) and VEGFD, VEGFA is the more potent, with VEGFA potentiating sponge angiogenesis from erythrocyte-containing vessels (visualised with H&E) per microscope field when dosed at 2.5 ng intrasponge twice daily, while VEGFD reached a maximum at 200 ng [135], possibly indicating activity through a different receptor. VEGFA induced angiogenesis, as assessed by ^{133}Xe clearance, and was inhibited by protein kinase inhibition with lavendustin A [112]. CP-547,632, a FGF-2/VEGF2 receptor kinase inhibitor reduced FGF- and VEGFA-induced sponge Hb content [136].

Whereas VEGF is universally known to be the most potent and selective EC mitogen, IL-1β appears significantly more potent in inducing corneal angiogenesis, requiring as little as 1 ng [137]. TNF-α has both stimulatory and inhibitory effects on angiogenesis, which are dependent on concentration and the system used. For example, TNF-α induces EC migration and tube formation *in vitro* [138], but *in vivo* 3.5 ng potently induces corneal angiogenesis; doses above this are inhibitory [139]. Sponge granuloma angiogenesis was accelerated by both IL-1β and TNF-α, but at high doses (50 ng), and this was inhibited by an IL-1 receptor antagonist and anti-TNF, respectively [140].

PDGF, the only angiogenic factor found in platelets, strongly potentiates sponge granuloma and freeze-injured skin graft angiogenesis, without appearing to have effects on EC DNA synthesis [141]. Site-directed mutagenesis and neutralising antibodies show that this effect is dependent on the thymidine phosphorylase active site of the molecule.

FGF-2 administration enhances the angiogenic response, and this has been investigated for a variety of interrelated mechanisms. Majita and colleagues [142, 143] first detailed the important role of Cox-2 and the dependency of FGF-induced sponge angiogenesis on it. FGF administration into the sponge induces increases in Hb concentration, granuloma development (measured as wet mass), a 13-fold rise in the synthesis of PGE_2 and 9-fold for 6-keto-PGF1α, and heightened VEGF mRNA expression. Both Cox-2 and VEGF expression peak at day 10, Cox-2 being expressed by the neovasculature. The prototype Cox-2 inhibitor NS398, when given prophylactically for 14 days, substantially reduced the Hb concentration within the sponges, as did indomethacin. These two drugs also completely inhibited FGF induced angiogenesis, as did the putative Cox-2 inhibitor [144] nimesulide. Granuloma development was only inhibited by indomethacin, however. It should be noted NS398 has a very short half-life, and required dosing three times daily, but while this regime still leaves overnight coverage incomplete, the inhibition of FGF-stimulated PG synthesis was completely abolished [143]. Both PGE_2 and beraprost (a stable PGI_2 analogue) induce sponge angioenesis to levels comparable to FGF alone.

The role of Cox-2-derived PGE_2 in sponge angiogenesis involves the induction of cAMP and protein kinase A (PKA) with the subsequent induction of VEGF [133, 142, 143, 145]. The PGE_2 and EP_4 analogues PGE(1)-OH and ONO-AE1-329 (5 nmol/sponge twice daily) promote sponge angiogenesis [146]. Interestingly, in studies *in vitro*, these agonists appear to act *via* the Erk and not the PKA pathway, contrary to that reported in the sponge model *in vivo* [133, 145]. However, another study reported that the EP_3 receptor was the main PG receptor, with the EP_3 agonist ONO-AE-248 being maximally effective, while EP1 (ONO-DI-004), EP2 (ONO-AEI-257) and EP4 (ONO-AEI-329) stimulation was reported to be less effective or ineffective at equal doses (10 or 30 nmol/sponge twice daily) [146]. The difference between these two studies could be in the end points, with the detection by histological means (rhodamine-dextran-positive structures per field) [146] as opposed to sponge Hb content [147]; as related earlier Hb reflects patent vascular volume, and rhodamine-dextran sulphate includes angiogenic pre-capillaries. However, EP_3-knockout mice do not respond with a good Hb/vWF sponge angiogenic response [147], and express low VEGF in the granuloma margins.

Evidence is now strong that substance P has a role in inflammation in angiogenesis. Substance P is found within rheumatoid synovial fluid, and substance P-containing nerve fibres innervate synovium but are depleted in rheumatoid pannus [148]. Even more importantly, they innervate the point of synovial insertion at the cartilage-bone interface (see this issue, and [149]). This is the area in which Fassbender

noted that the first sign of pannus formation is angiogenesis, followed by inflammatory cell recruitment [150]. Substance P is angiogenic in the rabbit cornea model while modulating EC function [151]. Substance P-containing neurones are present on microvessels supplying sponge granulomas, and NK1 receptors are present on the proliferating ECs surrounding the sponge [152] and their antagonism with selective NK1 antagonism, as opposed to NK2 and NK3, effectively inhibits substance P angiogenesis [110]. Such data support the observations of symmetrical development of rheumatoid disease, and the absence of disease in hemiplegic limbs. The application of NK1 antagonists, or other modulators of C fibre function, early in rheumatoid disease may therefore prevent the insidious advance of RA from joint to joint.

A variant of the granuloma has been used to investigate cannabinoid receptors, namely the carageenan sponge [153]. The addition of carageenan results in a substantial increase in inducible NOS (iNOS) and VEGF mRNA expression over control sponges, with small (~10%) increases in Cox-2 and TNF-α mRNA, whereas protein expression in all four is dramatically increased. Counts of H&E-stained vessels per field also more than doubled with the administration of carageenan. Cannabinoid agonism (WIN 55,212-2) is angiostatic, and the effect is mimicked by the CB(1) (arachidonyl-2-chloroethylamide) and CB(2) (JWH-015) agonism, each being reversed by the selective antagonists SR141716-A and SR144528 [153]. WIN 55,212-2 inhibits carageenan-stimulated inducible enzyme synthesis, i.e. iNOS and Cox-2, *via* the inhibition of NF-κB, as well as TNF and VEGF synthesis. Hence, these agents may have actions in addition to angiogenesis suppression.

IL-1-enhanced angiogenesis is inhibited by selective bradykinin receptor inhibition [86, 142]. This links to the kinin system and has created interest as the Hb content of rat sponges is reduced in kininogen deficiency [154], and suppressed by bradykinin B1 and B2 antagonism. The enhancement of IL-1-induced angiogenesis in the sponge is antagonised by B1 receptor antagonism, and not B2 [86].

Murine chronic granulomatous air pouch

The Freund's complete adjuvant/croton oil reaction has a component that induces a profound and enhanced angiogenic response. As a word of caution, the injection of the Freund's complete adjuvant without the croton oil induces an avascular granulomatous reaction [27, 92], which means that granuloma development does not require the angiogenic component to develop, an important point when considering the action of potential angiostatic agents. Thus, this model assesses the ability of agents to modulate inflammatory angiogenesis; however, in the absence of a concomitant alteration in granuloma mass, another model should be used to determine the disease-modifying potential of the treatment.

Our laboratory and others have utilised the murine chronic granulomatous air pouch to some degree to investigate the pharmacology of inflammatory angiogen-

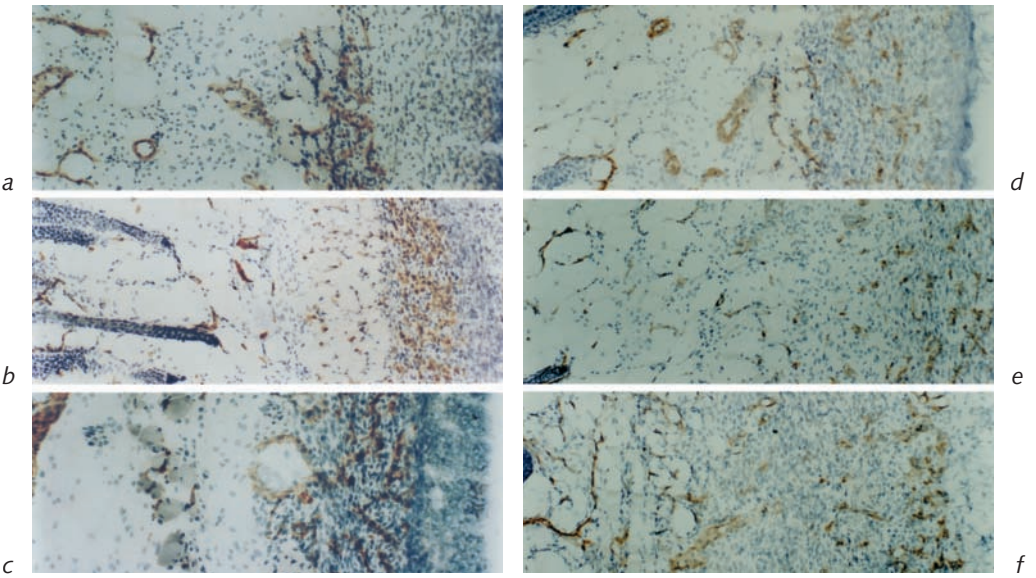


Figure 5
The development of the murine chronic granulomatous tissue air pouch vasculature as assessed by CD31 immunohistology [26], 15-μm sections counterstained with H&E. (a) and (b) show intense angiogenesis by days 3 and 4 derived from the subcutaneous skeletal vasculature, which develops into a dense tortuous capillary bed by days 5 and 6 (c, d, respectively). The tissue depth increases at 7 days and the capillary density reduces into vessels feeding the angiogenic front (e). By 2 weeks (f), three regions are seen, with the first possessing larger vessels, some appearing venular, followed by two regions corresponding to days 7 and 3.

esis, and the methodology is described by Alam [155]. The murine chronic granulomatous air pouch is more complex than the sponge granuloma. At days 3 and 4, the histology of the murine granulomatous air pouch is characterised by a densely packed collection of inflammatory cells (approximately 30–60 cells thick) predominantly polymorphonuclear neutrophils with some evidence of the presence of macrophages and fibroblasts. Staining for CD31 reveals early capillary development (Fig. 5a, b), derived from the established vasculature supplying the subcutaneous skeletal muscle. By days 5 and 6, this develops into a dense tortuous capillary bed (Fig. 5c, d) and by 7 days post induction the tissue depth has increased to approximately 150–200 cells. Layered variations in the cellularity can be discerned with that associated with the skeletal muscle being less densely packed than the rest of the tissue. This consists of fibroblasts and macrophages aligned and embedded in a network of collagen fibres (as determined by van Gieson‘s stain, not shown). The capillary density of this area is less than that of day 3 (Fig. 5e). The second area

extending to the pouch cavity is similar in composition to the day 3 lining, with a front of capillary development.

At 2 weeks after initiation, three regions are seen. The first consisting of a larger number of fibroblasts embedded in a densely packed extracellular matrix, along with macrophages and lymphocytes. The diameters of the vessels in this region are noticeably larger, some appearing venular. The two regions ventral to this are essentially similar to day 7 and day 3, respectively. From days 14 to 28, a further region developed with an area of large blood vessels surrounded by a mature fibrotic extracellular matrix, characteristic of new or granulation tissue that develops during wound healing. The capillary front remains present throughout, along with the extended vessels supplying it (Fig. 5f).

It can be seen that assessing this hyper-angiogenic response is difficult. The angiogenic front could be assessed as a 'hotspot', but this would ignore the remainder of the neovasculature in the deeper, as opposed to superficial, layers.

In addition, angiogenic capillaries develop from an arcade of mature subcutaneous vessels. Figure 1a shows a 4-day granulomatous air pouch. Vessels are clearly seen with an area of angiogenic granulomatous tissue development on either side. Figure 1d, shows a 6-day granulomatous air pouch; the granulomatous area is now wider and darker in appearance. The fronts merge to by day 14.

Mast cells are frequently seen in this model. Indeed, mast cells are often found in proximity to capillaries and release large quantities of heparin, which is pro-angiogenic in granulomatous tissue [26]. Thus, protamine antagonises granulomatous tissue angiogenesis [156] and, similarly, suramin antagonises the effects of heparin-binding growth factors in the sponge granuloma [134, 157].

Angiogenic and angiostatic responsiveness in this model has been demonstrated (Tab. 3). Increasing oral doses of Monoparin increase the vascular index whilst the angiostatic drug combination Monoparin/cortisone is suppressive. Interestingly, the angiostatic steroid tetrahydrocortisone (THC) is effective when given alone, combination with Monoparin is antagonising it.

Many of the factors that may be involved in this process are characterised. The murine granulomatous air pouch has been investigated for several of these factors [158]. Immuno-localisation studies have revealed that certain macrophages, but not all, express TGF-β, PDGF, TNF-α, IL-1α and IL-1β as would be expected. The proportion of macrophages and their contents increases with tissue maturity, except for PDGF, and are associated with areas of fibrosis. FGF-2 and TGF-β are present, bound to extracellular matrix, with FGF-2 becoming associated with ECs and basement membrane, whereas TGF-β is intense in fibrogenic areas and some fibroblasts after 14 days. ECs stain heterogeneously for EGF. The granulomatous air pouch thus expresses many angiogenic factors, localised to cells and matrix in such a way as to coordinate angiogenesis and wound healing. It is interesting to note the heterogeneous expression of factors within macrophages, ECs, fibroblasts and stroma, indicating focused local control.

Table 3 - The development of granulation tissue, vascular content (mg dye), and VI (μg/mg in mice treated with angiogenic or angiostatic stimuli); n=*8 per group,* ***p<*0.001*, **p<*0.01*, *p<*0.05 Mann-Whitney U test compared to vehicle control.*

Angiogenic	Dry mass (mg)	Dye (mg)	VI (μg/mg)	Angiostatic	Dry mass (mg)	Dye (mg)	VI (μg/mg)
p.o. vehicle	219 ± 28.8	0.77 ± 0.08	3.36 ± 0.53	p.o. vehicle	123.7 ± 18.4	0.89 ± 0.12	8.31 ± 1.46
100 U heparin	219 ± 12.0	0.90 ± 0.05*	4.24 ± 0.32	Cortisone (1 mg/kg)	120.3 ± 6.8	0.87 ± 0.08	8.23 ± 0.62
500 U heparin	214 ± 15.0	0.96 ± 0.04***	4.56 ± 0.48**	THC (1 mg/kg)	86.9 ± 8.7*	0.46 ± 0.03***	5.29 ± 0.61***
1000 U heparin	210 ± 16.0	1.03 ± 0.03***	4.84 ± 0.42**	Heparin (1000 U)	121.9 ± 17.2	0.84 ± 0.06	7.93 ± 0.83
2000 U heparin	215 ± 6.5	1.09 ± 0.05***	5.00 ± 0.50***	Heparin and cortisone	102.9 ± 13.0	0.69 ± 0.04 *	6.03 ± 1.12**
3000 U heparin	222 ± 7.5	1.23 ± 0.06***	5.60 ± 0.40***	Heparin and THC	78.9 ± 7.3*	0.48 ± 0.04**	7.11 ± 0.85
4000 U heparin	236 ± 11.5	1.42 ± 0.13***	5.97 ± 0.59***				
5000 U heparin	235 ± 10.6	1.94 ± 0.25***	8.53 ± 0.11***				

THC: tetrahydrocortisone

Cox-2 is expressed in the newly formed endothelium of granulomatous air pouches [159, 160] as well as in macrophages and infiltrating fibroblasts, with PGE_2 being the major arachidonic acid metabolite assayed. However, Cox-2 labelling is restricted to the subcutaneous venular endothelium, and is only observed in the neovascular capillaries from day 14 [159]. Its enzyme activity increases over 14 days, and is matched by protein expression [161]. NSAIDs and coxibs have a variety of effects on the development of the granulomatous air pouch [162, 163]. Aspirin at doses that inhibited Cox enzyme activity only had a small effect on tissue development at day 14, and was ineffective at altering carmine vascular index. Nimesulide, on the other hand, increased granuloma mass at day 14, and vascularity at day 7, while stimulating Cox activity at days 5 and 21, without effect at other time points. NS398 (10 mg/kg twice daily) tended to reduce the vascular volume, and had no effect on the other parameters at day 7. These effects are without any changes in Cox-2 activity, and it should be noted that the Cox enzyme assay reported here is an isolated enzyme assay that reflects enzyme content/activity, and not the degree of enzyme inhibition by the systemic administration of the NSAID inhibitors (except possibly that with aspirin, which covalently binds the active site). This is because NSAID binding is reversible and dissociation would occur during enzyme extraction. These data are consistent with a role for Cox-1 before day 14 [161]. However, another report found an inhibition of both vascular index and granulomatous tissue development with NS398 when dosed once daily at 10 mg/kg [164], and dipeptidyl peptidase (DPP), a dual inhibitor, with a Cox-2/Cox-1 inhibitory ratio of <0.0014 [165], had the same action. Both reduced endogenous granuloma PGE_2 content by ~50%.

The expression of Cox-1 at the time these assays are performed, days 6/7, would be consistent with the data presented in Table 2 [90], where the dual/Cox-1 inhibitors indomethacin, ibuprofen and piroxicam (NS) all enhance the carmine vascular index. Although they all reduced the vascular volume, they all also tended to increase granuloma mass by >15%. It should be noted that PGE_2, as well as being angiogenic [166], is immunosuppressive and involved in other aspects of the immune response such as T cell class switching [167]. Cox-1 and Cox-2 inhibition in colorectal tumours have similar effects, i.e. Cox-1/dual inhibitors (e.g. diclofenac) inhibit Colon-26 carmine vascular index and subsequent tumour growth, whereas Cox-2 inhibition is ineffective, including the use of DFU, another prototype coxib [98, 168–170]. Part of the problem could be answered by the dual inhibition of both Cox-2 and 5-lipoxygenase (5LO). MeUCH9, a dual inhibitor [171], was shown to inhibit both granuloma development and carmine vascular index [172], but in this trial prostanoid synthesis inhibition was not assessed, although both PGE_2 and LtB_4 were inhibited in the zymosan air pouch along with TNF-α. However, 5LO inhibition with Zileuton has a moderate but not statistically significant inhibitory effect on carmine vascular index [173], so it may be that inhibition of both enzymes simultaneously is required.

There are a variety of other factors known to be affected differentially by the interactions of NSAIDs with Cox, not least the resolvins and aspirin-triggered lipoxins [174]. Aspirin-triggered-15R-lipoxin A4 inhibits the VEGF-induced carmine vascularity index by > 50% [175].

The generation of prostanoids by the induced Cox-2 is dependent on the availability of substrate. Phospholipase A_2 (PLA_2) releases both arachidonic acid and lyso-PAF, the precursor to PAF. The PAF inhibitor Ro-24-4736 reduces pouch carmine VI [173]. The 14-kDa PLA_2 is associated with the 5LO pathway, and 85-kDa PLA_2 with prostanoid formation. SB203347, an inhibitor of 14-kDa PLA_2 [173], inhibits LtB_4 and PAF synthesis in the granulomatous tissue and inhibits the vascular density while leaving the granuloma development intact. In this report, PGE_2 was below the detection limit but the alternative Cox product, the resolving PGD_2, was expressed, and not inhibited by SB203347, demonstrating the selectivity of action against the 14-kDa protein. It thus appears that PAF derived from 14-kDa. PLA_2 is an important angiogenic product of the phospholipase/arachidonic acid cascade in inflammation angiogenesis.

NF-κB is involved in the up-regulation of a wide variety inducible enzymes and inflammatory molecules, including Cox-2, iNOS, TNF-α, and IL-1. The inhibition of inducible enzyme induction with DTD, an inhibitor of LPS-induced NF-κB activation, inhibits granulomatous tissue development, carmine vascular index, PGE_2, IL-1 and TNF-α synthesis as well as Cox-2 mRNA expression. p38 MAPK is also an important kinase in the messenger pathway for cytokines, including IL-6 [176], Cox-2 [177], TNF-α and IL-1 [178]. SB220025 inhibits granulomatous tissue angiogenesis, illustrated by the carmine vascular index, and visualised by cedar wood oil cleared pouches [178]. This agent is also anti-rheumatic in the murine CIA, illustrating that the lack of granuloma development is not a negative predictor of disease-modifying activity (see above).

Other low molecular weight drugs, especially anti-rheumatic agents, modulate inflammatory angiogenesis, although whether this is a primary action, or subsequent to immunomodulatory actions, remains debateable. Gold salts and methotrexate were among the first that were suspected to have this property. Early work showed that both auranofin and aurothiomalate inhibited cotton pellet granuloma angiogenesis (Seed MP, Halford J, Rising TJ, 1986, unpublished data), and aurothiomalate effectively inhibits angiogenesis in the murine chronic granulomatous air pouch [90]. Chloroquine, methotrexate and D-penicillamine also inhibit inflammation angiogenesis (Tab. 2). The only common denominator of this wide range of pharmacological agents with the correspondingly large number of molecular targets could be the ability to inhibit inflammatory angiogenesis but, in reality, the inhibition of angiogenesis by these drugs could be downstream of their immunomodulatory actions.

VEGF features strongly in this model, as in others, of inflammatory angiogenesis [179]. It is present in the first days of the reaction, up to day 3, reducing thereafter.

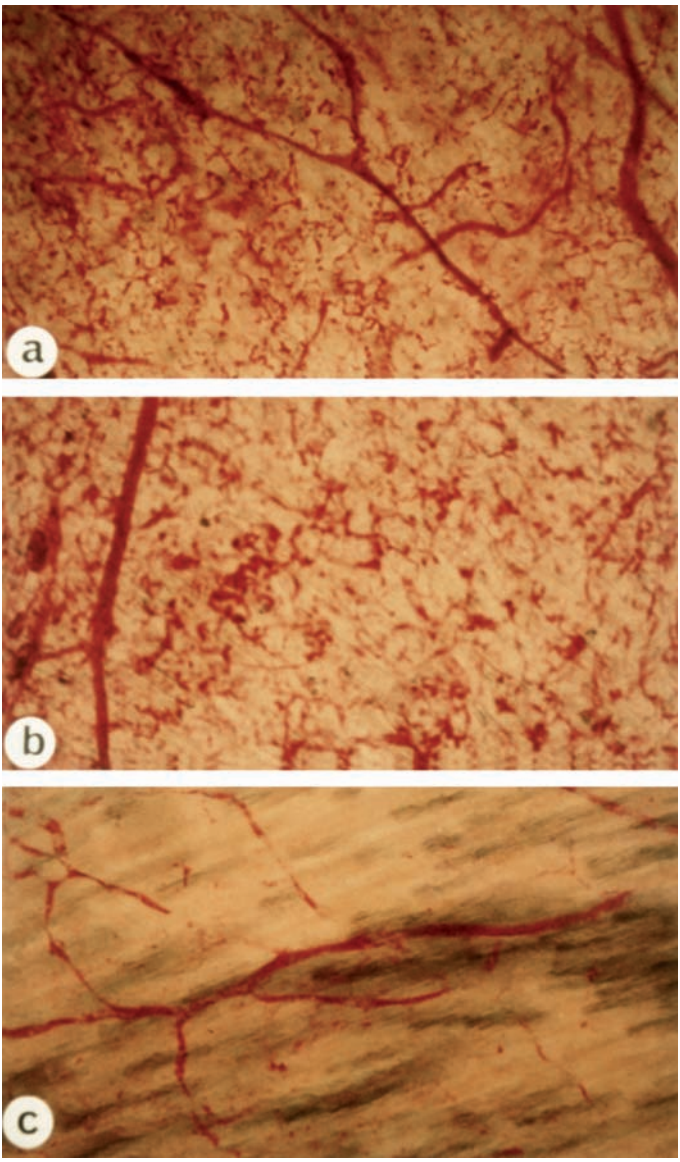


Figure 6
The inhibition of murine chronic granulomatous tissue angiogenesis by treatment with anti-VEGF [179]. The 7-day air pouches were cleared with cedar wood oil to reveal carmine vascular casts. Mice were treated i.p. twice with PBS (a), IgG control (b), or anti-VEGF (c). These data were confirmed by immunohistology with CD31, and carmine assay (PBS: 7.8±0.08; IgG: 7.0±0.9; anti-VEGF: 4.2±0.7 μg carmine/mg dry mass, $p<0.001$, $n=15$*). Reproduced by kind permission from John Wiley & Sons Ltd.*

The administration of anti-VEGF into the air pouch substantially reduces the carmine vascular index [179], and CD31-staining vasculature. Cedar wood oil cleared vascular casts of day 7 pouches demonstrates this effect (Fig. 6). The pan-VEGFR receptor kinase inhibitor PTK787/ZK222584 (vatalinib) inhibits granulomatous tissue development up to day 7, with a less proportionate reduction in carmine dye content [180]. If the vascular index is calculated in this instance, an increase in vascular density is evident. This could indicate that this inhibitor has additional anti-inflammatory activity. Indeed, subsequent work has shown this drug also inhibits other class III kinases including PGDF receptor β tyrosine kinase, c-Kit, and c-Fms, [181, 182].

The development of selective VEGF tyrosine kinase inhibitors, such as the flk-1 antagonists [183], may have promise as anti-angiogenesis agents in rheumatoid disease since the tyrosine kinase inhibitor lavendustin-A inhibits the VEGF/FGF-2 stimulation of granuloma angiogenesis [179]. In addition, the Tie family of tyrosine kinase receptors appear to be expressed on ECs during angiogenesis, and inhibition of their activity by blocking kinase activity may result in anti-angiogenic activity [184, 185]. Other kinases may also be involved in the pathway for transducing angiogenesis signals. It has been shown that inhibition of p38 kinase will block angiogenesis in the air pouch granuloma model [178]. Clearly, more work is needed to define the therapeutic potential of a wide variety of kinases and their inhibition in angiogenesis.

Another putative vasoactive mediator of inflammation, NO, is produced in granulomatous tissue by the endothelium and macrophages through iNOS [160]. We have found that L-arginine, the NO precursor, stimulates granulomatous tissue angiogenesis by 83% ($n = 10$) on oral administration [160]. Plasmin is thought to be involved in the activation of MMPs such as gelatinase and collagenase. We have shown that the inhibition of plasmin with tranexamic acid is effective in inhibiting granulomatous tissue angiogenesis [10, 186], as do other serine proteinase inhibitors such as soybean trypsin inhibitor and ONO5046.

The granulomatous air pouch has thus been used widely to assess the modulation of inflammation angiogenesis by a variety of drugs with a view to identifying mechanisms of action, or confirming *in vivo* anti-angiogenic activity in the inflammatory locus. Natural products, including structure activity relationships, e.g. liquorice-derived flavonoids, triterpenes, synthetic derivatives of tetrandines, and Chinese remedies [187–190], have also been tested.

Models of polyarthritis

The progression of disease in murine CIA occurs concomitantly with that of angiogenesis [191, 192]. The difference in the method of assessment can be seen in these studies when using vWF-stained patent vessels as the end point. Using the synovial

vascular bed area index, a dramatic increase is seen from the time at which clinical scores of disease are observed, i.e. day 28. When expressed as total vWF-staining endothelial area, this measure detects activity earlier, between days 14 and 28 [191]. Clavel and co-workers, using GSL-1, found a positive correlation between clinical scores and angiogenesis, as well as between histological scores and angiogenesis [192].

A wide variety of pharmacological modulators of angiogenesis have been assessed in models of polyarthritis. The angiostatic steroid 2-methoxyoestrodiol is effective in murine CIA [193]. Endostatin, an archetypal inhibitor of angiogenesis, is also effective in ameliorating murine anti-collagen antibody-induced arthritis [194, 195], although angiogenesis itself was not measured. The fumagillin derivative TNP470 (originally AGM 1470) is also effective in models of arthritis, both adjuvant-induced arthritis and CIA. Peacock and co-workers [14] showed the inhibition of histopathological signs, including the reduction in neovasculature assessed by histology, without the suppression of the DTH reaction or circulating antibodies to collagen in rat CIA. The same was seen with adjuvant-induced arthritis [15], where again T cell function appeared unaffected. The effects of TNP470 are especially apparent when given as a combination therapy, e.g. with taxol. Like the angiomodulatory factors in inflammation, assigning the efficacy of TNP470 to angiogenesis inhibition has been paramount. There have been reports that it has other modes of action such as interference in T and B cell function [16–19]. The reports that the DTH, antibody response, and T cell function in these models remain unaffected are important in determining an angiostatic, as opposed to immune, mechanism of action. The analogue PPI-2458 may be more selective, as discussed earlier, since it has been designed against MeA2I as opposed to being a natural product. PPI-2458 in this report did not affect off-target immune cells functions. PPI-2458 (10 nmol) inhibited human umbilical vein EC (HUVEC) proliferation and tube formation, but did not inhibit RA synovial fibroblast cytokine release, LPS-stimulated HUVEC cytokine synthesis or responses at the same concentration [20]. This novel series is thus very interesting, but the degree of separation of these actions and their bioavailability related to angiogenesis in the synovium remains to be determined.

The intra-articular transfection or gene transfer of angiostatin, the 5-kringle domain 38-kDa derived fragment of plasmin [196, 197], reduces both CD31- and vWF-determined vascularity in CIA, as well as synovial hyperplasia and erosion [66, 198]. The 5-kringle domain derivative of angiostatin, K1-5 is also effective [199]. Transfection of the soluble angiopoetin-1 and -2 Tie2 receptor, ExTek, is also anti-erosive, and inhibits CD31-determined angiogenesis [200]. The angiopeitins have well-characterised roles in angiogenesis but, like VEGF, may have other actions, e.g. a role in the inhibition of neutrophil adhesion and recruitment [201].

VEGF is a continuing area of keen interest, with bevacizumab in clinical trial for angiogenesis suppression in colorectal cancer [202], albeit with central nervous system side effects. These side effects may relate to the withdrawal of low levels

of VEGF that are thought to be required for the maintenance of capillary integrity [203] in a subset of patients. Models of polyarthritis have been used to investigate the possible benefits of anti-VEGF therapy. VEGF expression, as well as its receptors Flk-1 and Flt-1, in the CIA-affected joint correlates with neovascularisation, and anti-VEGF serum inhibited the onset of disease, but not severity, on therapeutic administration [204]. The effect of gene transfer of VEGF on clinical inflammation and joint histopathology depends on the site of administration [192]. Clinical score related to VEGF synthesis, when given i.m., correlated well with joint histopathology, but if given intra-articularly, did not affect clinical score, despite intense vascularisation. Interestingly, in this instance, histopathological score of inflammation and erosion correlated with the increased synovial VEGF expression. It should be recalled that VEGF is a highly potent inducer of EC retraction and vascular plasma exudation, so systemic availability could thus have marked effects on the clinical presentation of the disease. Knockout of VEGFR-1/Flt-1 reduces histopathology and joint erosion, but not vWF-derived angiogenesis score [205]. In addition, a synthetic inhibitor, KRN951, is anti-rheumatic. The anti-rheumatic response to K/BxN arthritis in the VEGFR-1/Flt-$1^{-/-}$ mice can be attributed to actions on macrophage/monocyte function [205], illustrating the caution that needs to be taken conferring mechanisms of anti-inflammatory action to pharmacological agents in this field.

Angiostasis can be induced in rat adjuvant-induced arthritis, despite the presence of VEGF, i.e. after hyper-expression of joint IL-4 [67]. IL-4 hyper-expression induced a profound suppression of vWF/integrin-staining vessels as well as EC tube-forming properties and angiogenic cytokine/chemokine synthesis *in vitro*. It appears that the mast cell is important [206], certainly in the K/BxN model of arthritis, as assessed by $\alpha v\beta 3$ integrin expression using ^{18}F-labelled galacto-RGD and positron emission tomography. Indeed, the depletion of $\alpha v\beta 3$ integrin-expressing cells shows that these cells are important for the development of arthritis [207], and that this is a promising target for anti-rheumatic therapy.

Models of IBD

The pharmacological investigation of angiogenesis in murine models of IBD is in its infancy. Since the first identification of the presence of angiogenesis in IBD, such as Crohn's disease and ulcerative colitis [71], the obvious question has been whether IBD would respond to angiostatic therapies. An initial indication was a case study in which thalidomide induced remission in a patient with Crohn's disease [120], and reduced dextran sulphate sodium (DSS)-induced histopathology [28]. However, as related above, thalidomide suppresses TNF synthesis [120] and TNF-induced NF-κB activation [121].

CD40 and CD40L appear to play a role in the development of DSS-induced IBD angiogenesis, as assessed by CD31 staining and computer-aided analysis of

microvessel density. Using CD40- and CD40L-deficient mice, this was linked to a reduction in VEGF expression by the tissues, and *in vitro* assays of EC tube formation inhibition [208]. Oddly, the lack of TP-1 enhances CD31/MECA 32-expressing vessels in this model [209]. Targeting vascular integrins has proven very successful in murine models of IBD. αvβ3 and α5 integrin gene expression is up-regulated in the murine $CD4^+CD45RB^{high}$ cell transfer colitis model [28] The peptide α5β1- and αvβ3-binding antagonist [210], ATN-161 (Ac-PHSCN-NH_2), inhibits both disease progression and angiogenesis as assessed by $CD31^+$ EC/DAB1 nuclear stain ratio angiogenic index, as well as total gut CD31 content, assessed by gut ^{125}I-labelled anti-CD31 binding, and *L. esculentum* lectin angiogenic index [28]. In IBD developed in non-barrier IL-$10^{-/-}$ mice, ATN-161 has also featured well, reducing both the disease activity index and angiogenesis as assayed through CD31-derived mean vascular density [8].

The induction of neovascular regression in chronic inflammation

The ultimate promise of the therapeutics discussed in this chapter must surely be not only the arrest of angiogenesis, but also the induction of neovascular regression. This occurs as a physiological consequence of wound healing and is demonstrated in the sponge granuloma [211]. CD31 analysis of the sponge granuloma reveals a peak of angiogenic activity at 24 weeks, with the peak of VEGF content at 6 weeks, and declining thereafter. This is an active process, and appears independent of the levels of pro-angiogenic factors present. Thus, the administration of VEGF at week 20 induces angiogenesis, but this effect ceases at week 24, and is ineffective thereafter during the regressive phase. This is also true for PDGF, FGF, and the combination of FGF/PDGF. The factors involved in this process are at present unknown. However, neovascular regression can also be induced pharmacologically.

This concept has been demonstrated in murine CIA [207]. The depletion of the synovial αvβ3 integrin-expressing neovasculature induced a regression of the clinical manifestations of the disease. This study shows the potential that may be had by the induction of neovascular regression, and the importance of angiogenesis in the maintenance of rheumatic disease development in these models.

The withdrawal of growth factors induces apoptosis in ECs [212], and similarly the withdrawal of VEGF from an angiogenic system in the eye results in capillary apoptosis and regression [213]. Cyclosporin administration to FGF-stimulated angiogenesis in Matrigel plugs also causes the regression of angiogenesis [214]. Cyclosporin is known to have negative actions at high doses against the microvasculature. However, in a more classical mode, the dosing of mice bearing established 7-day chronic granulomatous air pouches with the angiostatic combination cortisone/heparin results in not only the cessation of neovascular growth, but also its regression [215], similar to that seen in tumours (Fig. 7). Indeed,

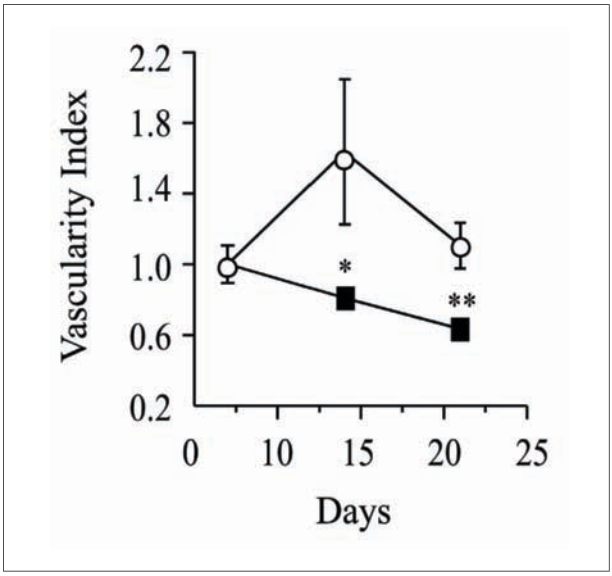


Figure 7
Neovascular regression in chronic inflammation induced by therapeutic, as opposed to prophylactic, dosing, started from 7 days after the induction of chronic inflammation in the murine chronic granulomatous air pouch with: the angiostatic 1 mg/kg cortisone/1000 U heparin combination s.c. (closed symbols), and vehicle controls (open symbols).

this has also been achieved by delivering high quantities of diclofenac locally to the air pouch [215, 216] using hyaluronan as the delivery system. Prostaglandin synthesis is reduced to levels half of those achieved by oral administration of the same dose (6 mg/kg), which is ineffective. This regression probably occurs as a result of EC apoptosis.

A hierarchy for the interaction of factors in inflammation angiogenesis?

It is apparent that inflammation angiogenesis in general is not the result of the action of one factor. Angiogenesis in inflammation is stimulated and maintained through the recruitment of angiogenic factor-secreting cells, as well as increased vascular permeability, the first and essential step. Once inflammatory cells are recruited, a panoply of cellular and cytokine/growth factor interactions occur, of which only a few have been demonstrated.

Synergism can be demonstrated, e.g. sub-threshold doses of VEGF and FGF-2 synergise in the sponge granuloma [179], and the same occurs with VIP and IL-1α.

However, AII causes intense angiogenesis without synergism with IL-1α. The strong activity of p38 MAPK inhibitors [178, 217] suggests that TNF-α and IL-1 may play a large role, but the angiostatic steroids act without modulating their synthesis, meaning either that these cytokines are irrelevant as far as angiogenesis in inflammation goes, or, more probably, that the angiostatic steroids are acting on factors or intermediates down-stream of the cytokines.

As already stated, VEGF expression is in turn stimulated by PGE_2, IL-1 and IL-6 [218, 219]. NO and/or the PGs or PAF may be the final mediators of some pathways. VEGF stimulates EC proliferation in an NO-dependent fashion [220] as well as vascular permeability *in vivo* [221], while FGF-2 -stimulated angiogenesis is sensitive to Cox inhibition [222]. However, reports on the modulation of NO synthesis and action in the chick chorioallantoic membrane assay and tumour angiogenesis models are contradictory. This may reflect the differing angiogenic growth factor milieu within these systems, VEGF-dependent systems being more reliant on NO, as are the PGs and substance P [151, 216, 220, 221]. Thus, in the case of FGF-2, the pathway may involve angiogenic actions of PGs acting *via* NO. The neovascular response to substance P and IL-1 in inflammation does not appear to involve the production of PGs, PAF or histamine since indomethacin, WEB-2086, mepyramine and cimetidine are not effective in antagonising them [110].

Summary

It has been shown over the last two decades that angiogenesis is an important aspect of tumour growth. More recently, attention has been paid to other proliferative diseases, typified by chronic inflammatory diseases, such as psoriasis and RA. This review has summarised the recent progress in the development of *in vivo* models to study inflammatory angiogenesis, the angiogenic/inflammatory factors produced in such diseases and models, and the potential benefits and difficulties in inhibiting inflammatory angiogenesis. Further clinical and preclinical experimentation will determine the role that angiogenesis inhibition could play in preventing, or even reversing, chronic inflammatory diseases.

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Angiogenesis in the inflammation of arthritis

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Introduction

Arthritis is a major source of pain, distress, disability and lost productivity to many members of society. People may be afflicted by various forms of arthritis. Osteoarthritis (OA) is almost universal in older people, with one joint or more being affected in almost everyone by the age of seventy. A process similar to OA can affect the spine (spondylosis) and is associated with back or neck pain. Rheumatoid arthritis (RA) affects 2–3% of western populations. Other forms of arthritis include the seronegative spondarthropathies associated with psoriasis, inflammatory bowel disease, anklosing spondylitis and reactive to infections. These each occur in smaller proportions of the population, but have major consequences for those who are affected. Currently available treatments help to control symptoms and may even limit the progression of joint damage, but none has so far been proven to cure joint disease. As with other chronic, incurable diseases, the burden of arthritis increases in ageing populations.

The classification of arthritic disease has evolved over the past century in response to the recognition of distinct clinical features. Historical classification of arthritis as either atrophic (e.g. RA) or hypertrophic (e.g. OA) has been refined. It is now recognised that different forms of arthritis have a predilection for different joint groups, may be associated with differing degrees of systemic inflammatory response, or with other concurrent diseases, and may have distinct genetic associations. In the absence of any clearly defined single causative agent for most forms of arthritis, aetiological or pathological classification of arthritis has not proven so useful in determining treatment as it has, for example, in pneumonia. Pathological or genetic features such as the presence of rheumatoid factors in the blood, of erosions on radiographs, or of the so-called human leukocyte antigen shared epitope are useful indicators of prognosis, even though, in isolation, they cannot precisely indicate diagnosis.

The earlier classification of arthritis as either atrophic or hypertrophic has stood the test of time. Atrophy of the joints occurs in RA. This is due to an aggressive

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growth of the mesenchymal tissues that line the joint, which becomes adherent to the adjacent articular cartilage, releasing enzymes which degrade cartilage and bone to form erosions. The release of inflammatory cytokines such as interleukin (IL)-1 from the synovial lining enhances bone resorption, leading to periarticular osteoporosis. The resulting atrophic appearance that is seen on plain radiographs in RA contrasts with the hypertrophic appearances in OA. In OA there is new growth of bony osteophytes around the joint margin, and thickening of the subchondral bone plate.

A clinical classification of arthritis as either inflammatory or non-inflammatory has facilitated the development of effective anti-inflammatory treatment strategies for some patients. No one would now question the historically established contribution of inflammation to RA, but the labelling of OA as a non-inflammatory arthritis may have concealed an important role of inflammation in its pathogenesis. It is now well recognised that patients with OA frequently complain of inflammatory symptoms such as joint stiffness, warmth and tenderness around the affected joint and swelling of the soft tissues of and fluid accumulation within the joint. Patients with OA not infrequently describe acute inflammatory flares of their joint symptoms, believed to be precipitated, in part, by calcium crystal depositions [1].

Histological demonstration of inflammatory cell infiltration into the synovial lining of the osteoarthritic joint is well established. Population studies have demonstrated that systemic markers of inflammation, such as C-reactive protein, are elevated in OA compared with non-arthritic controls, although not usually to the extent found in patients with RA [2, 3]. Anti-inflammatory strategies such as cyclooxygenase inhibitors and intra-articular glucocorticosteroids are of clinically proven value in relieving the symptoms of OA. Anti-inflammatory agents have been found to relieve symptoms more effectively in some patients than do simple analgesics such as paracetamol [4]. Inflammation therefore may be a feature of all forms of human arthritis, but the precise nature and consequences of the inflammatory response may vary between the different diagnoses.

The distinction between atrophic and hypertrophic forms of arthritis is not explained by the severity of inflammation, since ankylosing spondylitis in man and adjuvant arthritis in rats, each characterised by severe inflammation and responding clinically to anti-inflammatory treatments, are both associated with marked new bone formation around the affected joints. It is now believed that differences in the molecular regulation of inflammation may explain the varying pathological features of different arthritides. In this chapter we discuss how angiogenesis may contribute to these varying responses of the joint.

Structure of the normal joint

Most joints are diarthrodial, meaning that they contain a fluid-filled cavity bounded by cartilage of the articular surfaces and by synovium, a mesenchymal tissue that

lines the soft tissues of the joint. A second type of joint, the synarthroses, comprises fibrocartilaginous condensations between adjacent bones, displaying no synovium or synovial cavity.

The synovium of the diarthrodial joint contains two more or less discrete layers: the lining layer, which is normally apposed to the articular cartilage, and the sub-lining, which lies between the capsular ligaments and the lining cells. Although often referred to as 'synovial membrane', the synovial lining is embryologically derived from mesoderm rather than from ectoderm, and has no basement membrane. Blood vessels in the normal synovium are of decreasing calibre and increasing numerical density from the sub-lining to the lining layer [5]. The high-density capillary plexus immediately beneath the synovial surface provides the main nutritional support to the adjacent avascular cartilage. The vasculature of the normal synovium is highly organised, and vascular function is tightly regulated by peptide and non-peptide factors derived from vascular and perivascular structures. These include pericytes, fibroblasts and unmyelinated sympathetic and sensory nerve fibres.

Marrow spaces in subchrondral bone are vascularised in the normal healthy joint. Their blood vessels are associated with perivascular sympathetic and sensory nerves [6, 7]. Vascularity in the subchondral bone changes with age, irrespective of the presence of diagnosed arthritis [8]. Subchondral vascularity in the femoral and humeral heads decreases steadily from childhood then increases after the seventh decade. These vascular changes occur in parallel to decreasing and subsequently increasing bone turnover. The subchondral vasculature also contributes to the nutritional support of normal articular cartilage. Interruption of the subchondral blood flow leads to progressive deterioration of the articular cartilage over a period of weeks or months, indicating the importance of the subchondral vasculature to homeostasis in the joint [9].

Articular cartilage predominantly comprises a complex matrix of type II collagen, water and the proteoglycan aggrecan. Isolated chondrocytes, sparsely distributed within this matrix, constitute the sole cellular content of normal articular cartilage. Despite their low metabolic activity and normally hypoxic environment, chondrocytes maintain the matrix composition of the cartilage, while the matrix imparts elasticity and tensile strength. The deepest layer of articular cartilage adjacent to the underlying subchondral bone is calcified (but not ossified). The junction between the calcified and non-calcified cartilage is easily identified in histological preparations and referred to as the tidemark.

Normal adult non-calcified articular cartilage is avascular. The tidemark is believed to be an important defensive line in the articular cartilage and is rarely breached by blood vessels in the normal adult joint. Prior to the development of the tidemark, the deepest layers of the articular cartilage contain blood vessels originating from the subchondral bone. Furthermore, blood vessels are often localised to the calcified cartilage in post-mortem adult material [10]. The presence of some vascular channels within the calcified cartilage almost certainly represents normal

morphology. However, given the high prevalence of OA, it is not clear whether their observed frequency reflects normality, or whether post-mortem samples with large numbers of vessels in the calcified cartilage are from patients with subclinical arthritis. Penetration of blood vessels across the tidemark into the non-calcified cartilage is a typical feature of various forms of arthritis.

Joints are stabilised by surrounding ligaments and moved by muscles transmitting forces through adjoining tendons. Ligaments and tendons normally contain few blood vessels, comprising instead a tough matrix maintained by sparse fibroblasts. Dense vascular plexi coat the surfaces of these structures, providing their nutritional support. Ligaments merge with the vascular periosteum at the margin of the articular cartilage, a site known as the enthesis. The synovium adjoins the articular cartilage internal to ligaments at this point. The normal enthesis is highly vascular and well innervated. Meniscal cartilages in knees and temporomandibular joints are vascularised at their periphery [11, 12]. Injured and so-called degenerate menisci display increased vascularisation, often associated with other features of inflammation such as fibrin extravasation or inflammatory cell infiltration [13]. It remains controversial, however, as to whether this represents a desirable repair process or harmful pathology.

Sites of inflammation in arthritis

Synovium

Most studies of joint inflammation have focused on the synovium or the synovial fluid that it produces. This emphasis not only reflects the accessibility of these tissues for their study, but also the primary role attributed to the synovium in some forms of arthritis. Indeed, the restriction of inflammation to diarthrodial joints in RA emphasises the importance of the synovium.

Synovitis has been the subject of intense investigation in RA and the seronegative spondarthropathies. More recently attention has focused on the often less intense synovitis observed in osteoarthritic joints. Histological evidence of synovial inflammation is characteristic of both early and late OA [14, 15]. The synovitis of OA typically appears less intense than in rheumatoid disease, although both conditions may be characterised by perivascular infiltration by T cells [15, 16] and by the production of IL-1 and -6, and tumour necrosis factor (TNF)-α [14, 17]. The synovium is a heterogeneous structure, and synovitis has a predilection for regions of the synovium that are adjacent to articular cartilage [18, 19]. Indeed, removal of the articular cartilage by total joint replacement surgery markedly ameliorates clinical synovitis in RA.

Synovial inflammation is far from a single event or homogeneous process. Different forms of arthritis are characterised by different varieties of inflammation at

different stages in their progression. RA is believed to be driven by predominantly specific immune responses, mediated by lymphocytes, although other inflammatory cells including macrophages and mast cells play key roles. In OA, lymphocytes may also be observed in the synovium, although macrophages are the predominant inflammatory cell type present. Acute crystal-induced arthritides are associated with neutrophil accumulation in the joint, and also macrophage infiltration. In gout, inflammation is induced by urate crystals. In pseudogout, often associated with OA, calcium crystals such as calcium pyrophosphate dehydrate (CPPD) activate inflammatory cells.

Pannus

An important pathological feature in RA is the formation of synovial pannus (Fig. 1A). The word pannus describes a macroscopic cloth-like soft tissue that is often found covering the articular cartilage in RA. This invasive granulation tissue contributes to cartilage and bone degradation at the synovial-cartilage junction. Fibroblast-like type B synoviocytes congregate at the cartilage-pannus junction and produce matrix-degrading enzymes such as matrix metalloproteinase (MMP)-1, -3, -9 and -10 [20]. This leads to the erosion of the underlying articular cartilage. The growth of the pannus and its cartilage-degrading activity appears to be driven by factors such as IL-1β produced by macrophages and other inflammatory cells, coordinated in part by lymphocytes.

The formation of pannus is not confined to RA alone. A pannus-like tissue has also been described in a majority of joints affected by OA [21–23] (Fig. 1F). Similar pannus-like tissues have been described in spontaneous OA in mice [24], and in surgically induced OA in rabbits [25]. In general, fewer macrophages are observed in osteoarthritic pannus than in that found in RA [22]. However, an important contribution of inflammatory cells to its growth remains possible. As in RA, pannus in OA contains IL-1β- and MMP-expressing cells, suggesting a possible contribution to cartilage degradation [22, 23].

The enthesis

In contrast to RA, seronegative spondarthropathies, such as ankylosing spondylitis and psoriatic arthritis, affect synarthroses as well as diarthrodial joints. Indeed, the primary inflammatory lesion in these conditions appears to be at the highly vascular entheseal attachments of ligaments and tendons. Inflammation at non-synovial regions in the sacroiliac joints, vertebrae and ligament and tendon insertions can be associated with severe pain and disability. In addition, progressive new bone formation at these sites may eventually lead to bony fusion and

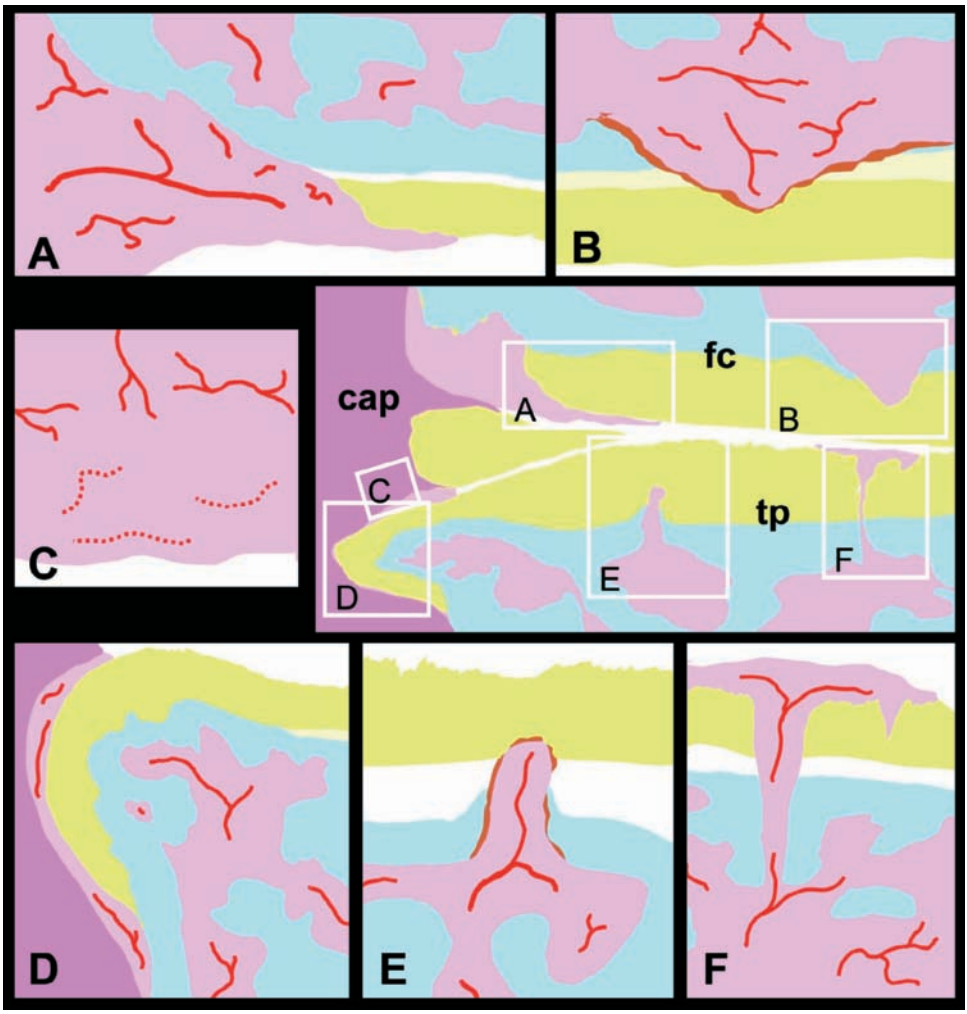

Figure 1
Sites of inflammation and angiogenesis within the arthritic joint. Central field: Schematic diagram of the medial compartment of a knee illustrating sites of angiogenesis during rheumatoid arthritis (RA) (fc: femoral condyle) and osteoarthritis (OA) (tp: tibial plateau). Cream: Non-calcified cartilage; green: bone, separated by calcified cartilage; pink: soft tissue of synovium, subchondral bone spaces and periosteum, each of which may be infiltrated by inflammatory cells; red lines represent blood vessels. Cap: joint capsule. (A) Pannus grows from synovium at the joint margin, adheres to and erodes underlying cartilage and bone in RA. (B) Inflammatory tissue within bone spaces invades into subchondral bone and deeper layers of articular cartilage in RA, behind a layer of clastic cells (brown). (C) Blood vessels proliferate within the deeper synovium, while those at the articular surface regress (broken lines), resulting in vascular redistribution, both in OA and RA. (D) In OA, osteophytes develop

total loss of movement. Suppression of inflammation by inhibiting TNF-α has led to major symptomatic benefit for patients with these conditions.

Subchondral bone

Inflammation has also been noted in the subchondral bone of joints affected by RA, even though this tissue is less easily accessible for study than is the synovium [26–28]. In non-arthritic joints, subchondral marrow spaces contain adipose tissue, often packed with haemopoietic cells (Fig. 2A). In RA these spaces are occupied by macrophage-rich fibrovascular tissue, often containing lymphoid aggregates (Fig. 2C). Inflammatory cell infiltration in the subchondral bone has also been noted in seronegative spondarthropathies [29]. In OA the subchondral marrow spaces are often filled by a less cellular, vascular granulation tissue containing some macrophages (Fig. 2B).

These inflammatory tissues may lead to an up-regulation of osteoclastic activity in this region of the joint with subsequent subchondral bone erosion (Fig. 1B) [28]. Erosion of the subchondral bone can lead to the formation of channels extending from the subchondral marrow space into the articular cartilage. Subchondral bone space contents such as resident vasculature and inflammatory cells can therefore gain access to the articular cartilage *via* this route. These observations have led to the concept of bidirectional damage to the rheumatoid joint, with erosion of the cartilage and bone occurring from the synovial pannus above and from the inflammatory subchondral tissues below. The subchondral inflammatory tissue appears to arise from bone marrow rather than through invasion by the synovium.

Synovial angiogenesis

Blood vessel growth in the synovium is consistently associated with synovitis [14, 30, 31]. Different patterns of vascularisation may reflect diverse regulation of angiogenesis between different forms of arthritis [32]. None-the-less, the link between angiogenesis and inflammatory cell infiltration is consistent across the disease spectrum from RA to OA. Furthermore, angiogenesis and inflammation are features of early, as well as late disease, both in RA and in OA [14, 31, 33–35].

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initially as cartilage outgrowths at joint margins, which then ossify through their vascularisation from the underlying bone spaces. (E) Vascular channels penetrate the subchondral bone plate into the deeper layers of articular cartilage in both OA and RA. Blood vessels grow into channels from the subchondral bone space, behind a leading edge of clastic cells. (F) Fibrovascular soft tissue occupies fissures in the articular surface in OA. Blood vessels within this osteoarthritic 'pannus' may originate from synovium or subchondral bone.

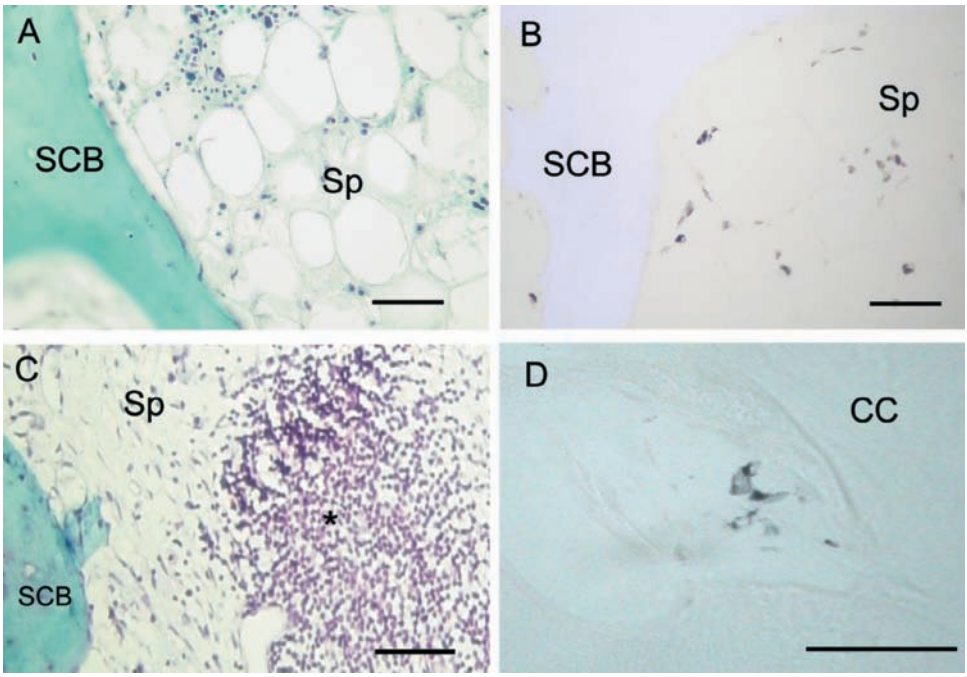

Figure 2
Osteochondral inflammation in human knees. (A) Subchondral bone from a post-mortem case with no evidence of arthritis showing fatty marrow with clusters of haemopoietic cells. (B) CD68-immunoreactive macrophages (black) in subchondral bone from a patient with OA. (C) Subchondral bone space occupied by fibrovascular tissue including a perivascular lymphoid aggregate (*) in a patient with RA. (D) CD68-immunoreactive macrophages (black) within a vascular channel invading the articular cartilage from a patient with OA. (A, C) Safranin O and haematoxylin stain; (B, D) CD68 immunoreactivity visualised using the ABC-peroxidase, nickel-enhanced diaminobenzidine method. Matrix components visualised by simultaneous autofluorescence with transmitted light. SCB, subchondral bone; Sp, subchondral bone space; CC, calcified cartilage. Bars = 100 μm.

There is a well-established reciprocal relationship between inflammation and angiogenesis. Inflammation can induce angiogenesis, for example through production of angiogenic factors by macrophages, while angiogenesis can facilitate inflammation through enhanced vascular permeability and inflammatory cell recruitment.

A wide variety of inflammatory mediators induce synovial angiogenesis in animal models [36]. These include acute inflammogens such as carrageenan and kaolin and capsaicin [37, 38], as well as chronic inflammatory stimuli such as Freund's complete adjuvant and injection of type II collagen in previously sensitised animals [39, 40]. Similarly, intra-articular injection of specific mediators will induce angio-

genesis, including the peptides substance P, calcitonin gene-related peptide and bradykinin, and polypeptide growth factors such as basic fibroblast growth factor (bFGF) [37, 38, 41, 42]. These studies confirm the potential for specific angiogenic factors to contribute to vascular growth in the synovium, and they provide useful experimental models for pharmacological studies. However, more sophisticated studies are required to demonstrate which inflammatory factors are those most critically involved in the pathological angiogenesis that occurs in arthritis.

The extent of synovial angiogenesis may vary between different forms of arthritis. Endothelial cells in newly formed capillaries within the rheumatoid synovium display higher indices of endothelial proliferation, and of the angiogenesis-associated Ets 1 transcription factor, than those in patients with OA [30, 43]. None-theless, some synovia in OA display a brisk angiogenic activity [30, 31]. Differences in vascular morphology suggest different mechanisms of angiogenesis between the various forms of inflammatory arthritis. For example, blood vessels observed by arthroscopy at the synovial surface appear to be more tortuous in psoriatic arthritis than in RA [32].

It may be expected that most anti-inflammatory strategies will inhibit inflammation-induced angiogenesis in the arthritic synovium [44]. Effective treatment by disease modifying anti-rheumatic agents or by anti-TNF therapy may each inhibit angiogenic factor production in patients with RA [45, 46]. These findings support the conclusion that, directly or indirectly, inflammation is a major cause of synovial vascular growth in RA, but cannot reveal the reciprocal role of angiogenesis in the disease process. Unfortunate, the availability of truly specific anti-angiogenic agents for use in man is somewhat limited. Clarification of roles for angiogenesis in the symptoms or consequences of arthritis depends on animal models, which may more or less reflect the processes that contribute to human arthritis.

The wide variety of angiogenic and anti-angiogenic factors that may be produced by synovial cells has been a subject of recent review [36]. *In vitro* studies have demonstrated that the angiogenic activity of synovial fluid from arthritic joints can be inhibited by blocking specific angiogenic factors, thereby implicating them in articular angiogenesis. Vascular endothelial growth factor (VEGF), TNF-α, hepatocyte growth factor, secreted phospholipase A_2, IL-18, fractalkine, and stromal cell-derived factor-1 each appear to make important contributions [47].

As with inflammation in other tissues, synovitis can induce angiogenesis. Inflammatory cells infiltrating the synovium may induce angiogenesis directly *via* release of angiogenic factors, or indirectly *via* stimulation of resident fibroblasts to produce similar factors. Resident or infiltrating cells within the synovium may be stimulated to produce angiogenic factors by both immune and non-immune mechanisms. CPPD crystals, associated with more severe OA, induce the expression of angiogenic factors such as TNF-α, IL-6 and IL-8 by monocytes and macrophages [48–50].

Macrophages are also the major source of the proinflammatory cytokine IL-1β. IL-1β, in turn, stimulates the release of angiogenic factors VEGF and PGE_2 from

fibroblasts that have been cultured from synovia of patients with either RA or OA [51]. In addition, it stimulates the production of matrix-degrading enzymes such as MMP-1 that may facilitate vascular invasion. IL-18 also induces VEGF production by rheumatoid synovial fibroblasts [51, 52]. Rheumatoid fibroblasts show higher levels of growth factor release than do osteoarthritic cells, consistent with a greater angiogenic stimulus in the rheumatoid synovium [53].

Other factors in the synovial environment may contribute to an up-regulation of angiogenesis. The inflamed synovium is maintained in a hypoxic environment [54]. Raised intra-articular pressure results from the force of muscular contraction on the expanded, incompressible synovial fluid. Intra-articular pressure may exceed capillary filling pressure, especially during exercise, resulting in capillary closure and hypoperfusion. Concurrently, the proliferating and inflamed synovium exerts increased metabolic demand and oxygen consumption. Hypoxia in the inflamed synovium up-regulates the expression of hypoxia-inducible factor (HIF)-1α [55–57]. In turn, hypoxia may facilitate the secretion of VEGF by synovial fibroblasts [51]. The angiogenic effects of cytokines are at least additive to those of hypoxia [51, 52]. Hypoxia in the inflamed synovium therefore potentiates a more direct stimulus to angiogenesis resulting from growth factor expression by inflammatory cells.

The rate of angiogenesis in the inflamed synovium, as indicated by the frequency of proliferating endothelial cell nuclei, is comparable to that observed in malignant tumours and during wound repair. Such a rapid proliferation rate and vascular growth would be inconsistent with homeostasis in the joint were it not balanced by equally brisk vascular regression. Histological studies have indicated that endothelial cell apoptosis is concurrent with vascular proliferation, but that apoptosis and proliferation occur in spatially distinct zones of the synovial tissue [30]. This suggests the differential but co-ordinated regulation of vascular growth and regression, both associated with the chronic inflammatory process. A wide variety of factors potentially modulates endothelial cell apoptosis, particularly transforming growth factor (TGF)-β [58, 59]. However, which of these or other factors are responsible for co-ordinating vascular turnover within the inflamed synovium remains to be determined.

Intra-articular injection of angiogenic factors is followed by synovitis [37, 42]. However, as most angiogenic factors are also pro-inflammatory, it has been difficult to prove whether this synovitis is a consequence of angiogenesis or a direct pro-inflammatory action. bFGF stimulates synoviocyte proliferation but has little effect *per se* on inflammatory cell recruitment. Intra-articular injection of bFGF stimulates angiogenesis and exacerbates existing synovitis, supporting the view that angiogenesis itself may potentiate synovial inflammation [42].

Pharmacological agents that inhibit angiogenesis also inhibit synovitis. However, again, the very close association between angiogenesis and inflammation makes it difficult to distinguish between anti-angiogenic affects and direct anti-inflammatory activity. Indeed, synergistic activity between anti-angiogenic and anti-inflammatory agents in modifying arthritis may indicate that combining these activities may

have particular therapeutic benefit [60]. Both angiogenesis and arthritis are each inhibited by integrin antagonists [42, 61], fumagillin derivatives [39, 40, 62–65], angiostatin [66–68], endostatin [69], IL-4 [70], IL-13 [71], kalistatin [72], thrombospondin [73], and agents that block VEGF signalling [74, 75], Tie-2 signalling [76] or urokinase plasminogen activity [77].

Increased vascular turnover in the inflamed synovium affects not only the vascular density but also its quality. Several studies have found that overall vascular density in inflamed synovium is unaltered or even reduced compared with the high vascular densities observed adjacent to the normal synovial surface [78, 79]. The total vascular volume undoubtedly is increased during synovitis, as demonstrated by magnetic resonance imaging, Doppler ultrasound, and by extraction of intravascular markers [80, 81]. However, this increase in vascular volume may be proportionate to the increase in synovial volume rather than indicating any increase in vascular density.

Synovial inflammation is associated with a re-distribution of blood vessels with reduced vascular density at the synovial lining and increased vascular density in the deeper sub-lining tissues (Fig. 1C) [78]. Blood flowing to the normal synovial lining provides important metabolic support to the adjacent avascular articular cartilage. Diversion of blood into the deeper synovial layers in fact may exacerbate articular hypoxia by reducing blood flow at the lining surface. Blood flow distribution within the synovium is determined not only by the localisation of blood vessels, but also by their reactivity [82]. Diversion of blood flow by redistributed blood vessels may be further aggravated by dysregulation of the synovial microvascular bed, as immature blood vessels display incomplete vasoregulatory systems. An inability to close blood vessels in the hypervascular deep regions of inflamed synovium may exacerbate blood diversion away from the synovial lining.

A further possible consequence of angiogenesis in the synovium, as well as in other parts of the inflamed joint, is exacerbation of pain. During acute synovitis inflammatory mediators such as prostaglandins and kinins sensitise peripheral nerve terminals, resulting in the activation of nociceptive pathways by what would normally be innocuous joint movements. Hypoxic metabolism and resulting acidosis within the joint may sensitise nociceptive pathways and cause pain even after inflammation has subsided. Vascular growth is followed by the growth of fine, unmyelinated sensory nerves along the new blood vessels [7, 83, 84]. Extending nerve terminals respond abnormally to physical and chemical stimuli, and their presence may also be associated with enhanced pain sensation.

Angiogenesis and pannus

The vascularisation of pannus is believed to be important for its continued growth (Fig. 1A). The synovial pannus in RA has been likened to a malignant tumour with

respect both to its growth and its invasive properties [85]. In the same way that tumour growth is limited by its vascular supply, so may be that of the synovial pannus. As synovial pannus grows and erodes (invades) underlying cartilage and bone, the articular surface is progressively damaged. Erosions, seen initially only by ultrasound and magnetic resonance imaging, become visible on plain radiographs and may eventually be associated with secondary osteoarthritic changes. Angiogenesis inhibition has been associated with reduced synovial volume and reduced erosive joint damage in some studies [42, 64, 68, 73, 76].

Osteoarthritic pannus-like tissues may also be highly vascular (Fig. 1F). Indeed, vascularisation of osteoarthritic pannus may be sufficient to permit its detection by colour Doppler ultrasonography [86]. The roles of osteoarthritic 'pannus' in symptoms or disease progression remain unclear, and the contributions of inflammation and angiogenesis to its growth are understood less well than for rheumatoid pannus. OA is not typically characterised by bony erosion, although an adverse effect of osteoarthritic pannus on cartilage homeostasis and a contribution to osteoarthritic cartilage damage is likely.

Pannus in OA is often in continuity with bone marrow, suggesting a role for the subchondral mesenchymal tissues in its generation [22]. Osteoarthritic pannus is also observed predominantly near the joint margin, suggesting, in common with rheumatoid pannus, a synovial origin [21, 22, 24]. Blood vessels within the growing pannus may therefore originate from either synovium or subchondral bone, and pannus represents heterogeneous tissues of different origins and, possibly, consequences.

Osteochondral angiogenesis

Both in RA and in OA, the articular cartilage loses its ability to remain avascular [87]. New blood vessels gain access to the articular cartilage, growing within channels that originate from the subchondral bone marrow spaces (Fig. 1E) [10, 88, 89]. These vascular channels extend to breach the tidemark into the non-calcified articular cartilage. New blood vessels within these channels are embedded within a cellular matrix, and the channels become surrounded by cuffs of new bone.

Vascular invasion of the articular cartilage is permitted by the creation of channels by osteon-like remodelling units. These are led by a tunnelling cellular front of chondroclast/osteoclastic cells (cutting cones) [89]. Cutting zones resorb extracellular matrix, creating channels into which blood vessels can grow.

Different terminologies have been used to describe aspects of these vascular channels as they develop and mature. Subchondral bone resorption pits [90], which appear as holes and indentations in the subchondral plate when viewed by electron microscopy [88], and calcified cartilage canals [91] probably represent beginning and end of vascular channel development.

The detailed regulation of osteochondral angiogenesis remains incompletely understood. Key features appear to be stimulation of blood vessel growth, and permissive changes within the articular cartilage that remove its normal hostility to vascular invasion. Inflammation may contribute to both of these processes.

It is likely that subchondral inflammation modulates osteochondral angiogenesis. Cellular infiltration of the subchondral bone marrow spaces in human OA is associated with osteochondral vascularisation. Vascular connective tissue proliferation was also detected at the subchondral plate in an animal model of OA [92] and was present during the repair of surgically induced osteochondral defects in goats [93]. Bone marrow replacement is associated with vascular invasion into the articular cartilage, and the subchondral vascular tissues appear continuous with that within the vascular channels of osteoarthritic cartilage. Cellular infiltrations actually within vascular channels in rheumatoid [26] and osteoarthritic [90] articular cartilage include macrophages, further suggesting a role of inflammation in vascular channel development (Fig. 2D). T cell infiltration and osteoclastic foci were also associated with subchondral angiogenesis in femoral heads from patients with ankylosing spondylitis [29]. The generation of angiogenic factors by inflammatory cells in the subchondral spaces and within vascular channels may stimulate blood vessel growth.

Inflammation may further facilitate osteochondral angiogenesis by modulating homeostasis of the articular cartilage. Cartilage is normally hostile to vascular invasion. Articular cartilage from normal joints is resistant to vascular invasion from chicken allantoic membranes, whereas that from patients with OA displays enhanced vascular invasion [87]. Changes in chondrocyte function and cartilage matrix composition that result from inflammation may be important in permitting vascular invasion.

The resistance of normal articular cartilage to vascular invasion is partly due to its content of anti-angiogenic factors such as tissue inhibitors of metalloproteases, and also partly by merit of its matrix composition. Osteochondral vascular invasion in OA occurs in association with glycosaminoglycan depletion in the articular cartilage [10, 87]. Vascular channels penetrate parts of the articular cartilage that are deficient in proteoglycans and glycosaminoglycans. Pro-inflammatory cytokines such as IL-1 stimulate, and anti-inflammatory glucocorticosteriods inhibit, the synthesis and activity of MMPs and other enzymes that facilitate cartilage matrix degradation in both RA and OA [94–96]. Subchondral inflammation and inflammatory cells within the channels themselves may contribute to these permissive changes in the cartilage matrix.

Other processes in the subchondral bone may contribute to osteochondral angiogenesis. Considerable circumstantial evidence links bone turnover and vascular growth. The foramina of vascular channels in racehorses are often lined up in rows, suggesting that they originated as side branches from a shared subchondral bone space [91]. The channels cluster where the subchondral bone density is low-

est, where large marrow spaces are close to the calcified cartilage. Vascular channels similarly penetrate the subchondral bone plate in humans in regions where bony trabeculae are widely spaced [88]. Osteoarthritic femoral heads have greater spacing between trabeculae than osteoporotic femoral heads [97], and this subchondral bone remodelling may itself be permissive for osteochondral angiogenesis.

Rather than being merely permissive, osteoblasts and osteoclasts may play more direct roles in the regulation of osteochondral angiogenesis, as suggested by an association between increased osteochondral vascularisation and increased bone turnover. Alendronate, a potent inhibitor of osteoclastic activity and therefore bone resorption, suppressed the vascular invasion of the calcified cartilage and reduced osteophyte formation in an animal model of OA. It is likely that Alendronate inhibited osteoclasts and chondroclasts in the cutting cones of vascular channels, thereby preventing vascular invasion. Some bisphosphonates, particularly those not containing amino groups, also have anti-inflammatory activity [98–100]. Inhibition of angiogenesis by bisphosphonates therefore may also be mediated by their anti-inflammatory actions.

The importance of osteochondral angiogenesis in the pathogenesis of arthritis, and the contribution of inflammation to that process, deserve further study. Vascular invasion of the articular cartilage may contribute to its degradation, altered biomechanics, ossification and innervation.

An important consequence of vascularisation of cartilage is bone formation. The invading vasculature, alongside periosteum and cartilage, is a key player in the initiation of endochondral ossification [101]. Vascular channels in arthritic articular cartilage are accompanied by the deposition of new collagen types, notably I and X, that are otherwise more characteristic of bone matrix [87]. The association of channels with cuffs of bone extending into the articular cartilage suggests that they contribute to bone turnover at the osteochondral junction [10]. Vascular channels may become filled with a cartilaginous matrix, which subsequently calcifies [91]. As the osteochrondral junction advances into the remaining articular cartilage in OA, the subchondral bone plate thickens.

Vascular invasion of cartilage occurs during the growth and maturation of long bones, even in the absence of inflammation. In this context chondrocytes are aligned in columns, hypertrophy and generate angiogenic factors, which in turn stimulate the growth of blood vessels into the channel left by the chondrocytes as they apoptose. Chondrocytes within the articular cartilage may also express markers of hypertrophy, such as collagen X (Fig. 3A). However, the appearances of vascular channels at the osteochondral junction in articular cartilage are different from those seen in the epiphyseal growth plate. Articular chondrocytes are typically not aligned in columns, but rather exist as discrete clusters or chondrons (Fig. 3). Blood vessel growth does not appear to be directed towards chondrons, but rather follows regions of glycosaminoglycan depletion. Angiogenic factor (e.g. VEGF) production by articular chondrocytes is particularly localised adjacent to the articular surface,

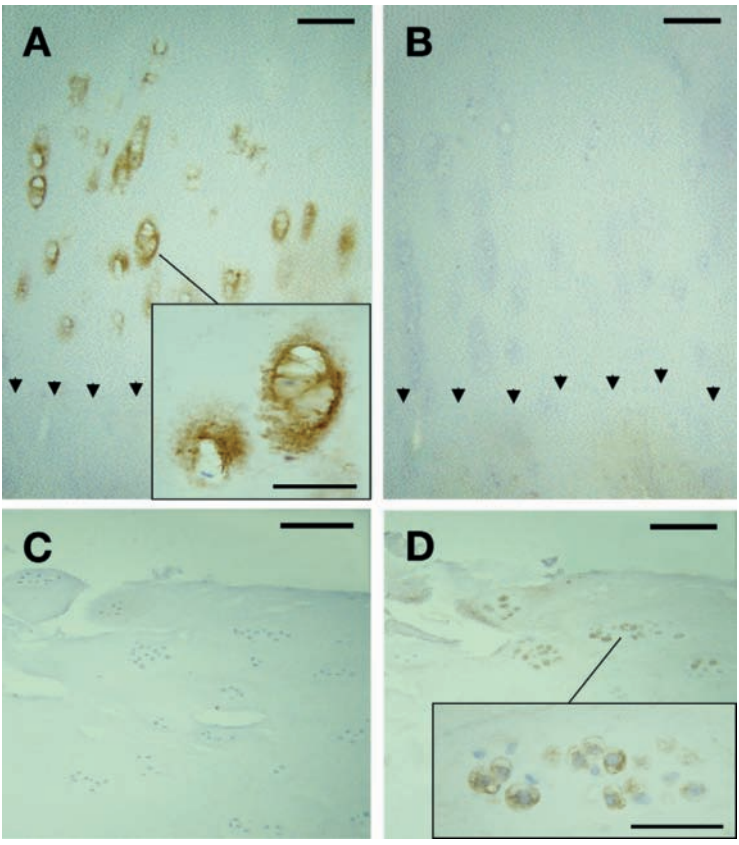

Figure 3
Collagen X and vascular endothelial growth factor (VEGF) immunoreactivity, markers of chondrocyte hypertrophy and angiogenic factor production, in the non-calcified cartilage of medial tibial plateau samples from patients with OA. (A) Collagen X-immunoreactive chondrocytes and chondrocyte clusters (brown) are present in the deep zone of the non-calcified cartilage, above the tidemark (arrows). (Inset) Higher magnification view of a single chondrocyte and chondrocyte cluster immunoreactive for collagen X, with collagen X present in the peri- and extracellular matrices. (B) A sequential section from the same sample stained for VEGF. VEGF is not produced by chondrocytes that express collagen X in the deep zone of articular cartilage. (C) Sample stained for collagen X showing an absence of collagen X-immunoreactivity in chondrocytes near the irregular articular surface. (D) A sequential section from the same sample stained for VEGF. VEGF-immunoreactive chondrocytes (brown) are present in the superficial zone of the cartilage. (Inset) Higher magnification view of a chondrocyte cluster and single chondrocytes immunoreactive for VEGF. (A, C) Prepared using monoclonal antibody clone X53 (Quartett Immunodiagnostika und Biotechnologie GmbH, Berlin, Germany) following antigen retrieval with type XXIV protease and type I testicular hyaluronidase. (B, D) Prepared using polyclonal antibody A-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Scale bars, main figures: 100 μm, inserts: 50 μm.

adjacent to the inflamed synovium, rather than at the osteochrondral junction where vascular channels occur (Fig. 3B, D). This indicates that the growth of vessels within the articular cartilage may be determined more by their immediate cellular environment than by the more distant chondrocytes. Therefore, although vascular invasion of articular cartilage may lead to its ossification, as it does in the epiphyseal growth plate, the regulation of angiogenesis may be very different in the two tissues.

The mechanisms by which vascularisation of articular cartilage leads to its ossification remain incompletely understood. All but the most immature blood vessels are surrounded by spindle-shaped or pleomorphic cells called pericytes. Pericytes themselves can differentiate into multiple cell types including osteoblasts and chondrocytes [102]. They express chondrogenic markers such as Sox9, collagen II and aggrecan. When cultured in high-density pellets in the presence of a chondrogenic medium that contained TGF-β3, pericytes deposited a cartilaginous matrix rich in proteoglycans and type II collagen that was very similar to that seen in the articular cartilage [103]. Furthermore, artificial tissues resembling bone, mineralised and non-mineralised cartilage as well as fibrocartilage developed in diffusion chambers containing pericytes that were implanted into athymic mice [103]. Pericytes therefore may be responsible for deposition of cartilage matrix seen within vascular channels at the osteochondral junction.

In contrast to this potential for neovascularisation to enhance bone formation, angiogenesis at the osteochondral junction may conversely be associated with bone and cartilage destruction. Indeed, subchondral inflammatory tissue in RA has been described as a form of 'pannus' and inflammatory angiogenesis that occurs in the pannus on the articular surface may be replicated in the subchondral bone. The articular cartilage therefore may be invaded from above by synovial pannus, and from below by a similar tissue originating from the subchondral marrow spaces [26].

The consequences of osteochondral angiogenesis await definitive elucidation by the use of specific anti-angiogenic agents. It is currently unknown whether vascularisation of the deeper layers of the articular cartilage is an appropriate response to hypoxia, in some way compensating for reduced vascularity of the synovium. Alternatively, vascular invasion of the cartilage may destroy the normally protective barrier between bone and cartilage, impair the cartilage's structural integrity, and exacerbate abnormal cartilage turnover through the release of cytokines and other factors by cells within the channel.

In addition to influencing joint damage, osteochondral angiogenesis may contribute to pain, the predominant symptom of arthritis in man. In the normal joint, nociceptive nerve fibres are absent from weight bearing articular structures such as the cartilage, as their presence would result in pain during normal activity. The blood vessels within osteochondral channels may be associated with fine unmyelinated sensory nerves, such that vascular invasion is associated with neoinnervation of the articular cartilage [7, 104]. Osteochondral angiogenesis may therefore con-

tribute to the chronic pain of arthritis. Inflammatory mediators within the vascular channels may further exacerbate pain by sensitising nerve terminals.

Angiogenesis, inflammation and osteophyte formation

Angiogenesis and inflammation are also associated with osteophyte formation in the osteoarthritic joint (Fig. 1D). Angiogenesis is a key step in endochondral ossification and the growth of osteophytes in the osteoarthritic joint is believed to be angiogenesis dependent [105]. Osteophyte growth is also associated with inflammation [106]. Progression of osteophytosis in lumbar spondylosis has been associated with polymorphisms in genes involved in inflammation and angiogenesis: MMP-3, tissue inhibitor of metalloproteinase-1 and cyclo-oxygenase-2 [107]. Injection of TGF-β into normal mouse knees induces a combination of inflammation, synovial hyperplasia and osteophyte formation [108]. Osteophytosis is accelerated in inflamed osteoarthritic joints, and anti-inflammatory doses of *N*-iminoethyl-L-lysine (L-NIL), a nitric oxide synthase inhibitor reduced the formation of osteophytes in a canine model [109]. A contribution of blood vessel growth to the association between inflammation and osteophyte formation, although plausible, remains to be proven.

Therapeutic implications

The bidirectional relationship between inflammation and angiogenesis has important therapeutic implications for the development of treatments for arthritis. Where angiogenesis is driven by inflammation, anti-inflammatory strategies may alleviate the adverse consequences of blood vessel growth. For example, suppressing the inflammatory drive to angiogenesis may permit normalisation of perfusion in the synovium, thereby relieving hypoxia. It may limit pannus growth and invasion, reduce bone turnover at the osteochondral junction, and prevent the growth of nerves in articular tissues and thereby reduce chronic pain. In addition, anti-angiogenic treatments that are not of themselves anti-inflammatory, may achieve all of the above and also relieve chronic synovitis by counteracting facilitation of inflammation by the new vascular bed.

Anti-inflammatory treatments suppress angiogenesis in the synovium. Indeed most, if not all, treatments that suppress synovitis also reduce vascularity in the synovium, consistent with the view that inflammation is the primary drive to synovial angiogenesis during arthritis. Locally generated and circulating angiogenic factors, such as VEGF, are decreased following anti-inflammatory treatments in patients with RA, indicating that they may mediate the inflammatory drive to synovial angiogenesis [45]. Specific molecular manipulation of angiogenic pathways in animal models of chronic inflammatory arthritis, either through traditional

pharmacological approaches or gene transfer, indicate potential but have yet to be translated into clinical benefit in human disease.

Whereas relationships between inflammation and synovial angiogenesis are now well established, it remains less clear whether anti-inflammatory strategies can also inhibit osteochondral angiogenesis and its consequences. Increased temperature and effusions in osteoarthritic knees have been associated prospectively with radiographic deterioration over the subsequent 1–5 years [110]. Furthermore, low-level increases in the systemic inflammatory marker C-reactive protein were associated with more rapid radiological progression of knee or hip OA [2, 111]. Synovitis induced by intra-articular injection of CPPD crystals exacerbated OA in an animal model [112]. Preliminary data indicate that blood vessel growth at the osteochondral junction depends more on local factors within the articular cartilage than on synovial inflammation, although this does not exclude an important contribution from subchondral inflammation. Molecular mechanisms that underlie osteochondral inflammation and angiogenesis remain poorly understood, and further work will be required to identify how these may differ from those in the inflamed synovium.

Preliminary work with corticosteroids indicated that doses of prednisolone that were sufficient to inhibit synovial inflammation, also decreased osteophyte size and cartilage ulceration in dogs with surgically induced OA, whereas lower doses did not [113]. Similarly, anti-inflammatory doses of the inducible nitric oxide inhibitor L-NIL decreased the size of cartilage lesions in a canine surgical model of knee OA [109]. Specific targeting of osteochondral inflammation and angiogenesis would help determine their contributions to the pathogenesis of arthritis, and how they may be distinct from additional, sometimes concurrent, effects from inflammation in the synovium.

Conclusions

Diverse anti-angiogenic strategies have been shown to reduce inflammation and joint damage in animal models of arthritis. Although for each therapeutic strategy parallel effects on disease that are independent of angiogenesis inhibition cannot be excluded, consistent findings across a wide variety of anti-angiogenic agents suggest that inhibition of blood vessel growth itself is therapeutically important. Although we can be optimistic that animal studies may be extended to man, therapeutic strategies in human arthritis have tended to focus on direct inhibition of inflammation. Consequently, the role of angiogenesis inhibition in their anti-inflammatory effects remains unclear. Of key importance will be whether anti-angiogenic strategies can reduce other symptoms and signs of inflammation; pain, stiffness and swelling. The development of anti-angiogenic treatments in non-rheumatological fields of medicine such as oncology raises hope for a group of conditions that are currently incurable and cause widespread disability and distress.

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